IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Robert H. Harris	**Examiner:**	David Lukton
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For:	NEW USES FOR AMINO ACID ANTICONVULSANTS		

Confirmation No: 2598

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF ROBERT H. HARRIS
UNDER 37 C.F.R. §1.132

Sir:

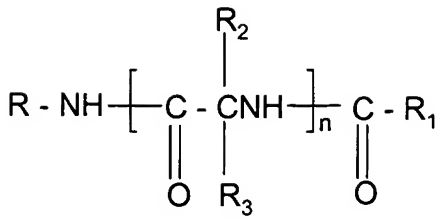
Dr. Robert H. Harris declares and says as follows:

1. I am the inventor of the subject matter of the above-identified application, and I have complete knowledge of all aspects of the invention embodied therein.

2. I have received the degree of the Doctor of Philosophy in Biochemistry from Rutgers University in 1977.

3. I currently am the owner of and hold the title of President at Harris FRC Corporation.

4. The present application is directed, inter alia, to the method of treating a patient suffering from bipolar disorder comprising administering thereto a therapeutically effective amount of a compound for treating bipolar disorder, said compound having the formula:



wherein

R is aryl lower alkyl and R is unsubstituted or is substituted with at least one electron withdrawing group or electron donating group;

R_1 is lower alkyl and is unsubstituted or substituted with at least one electron withdrawing group or electron donating group;

R_2 is hydrogen, lower alkyl, lower alkenyl, lower alkynyl, aryl lower alkyl, aryl, halo, heterocyclic, heterocyclic lower alkyl, lower alkyl heterocyclic, lower cycloalkyl, lower cycloalkyl lower alkyl, or ZY,

R_3 is lower alkyl, lower alkenyl, lower alkynyl, aryl, aryl lower alkyl, halo, heterocyclic, heterocyclic lower alkyl, lower alkyl heterocyclic, lower cycloalkyl, lower cycloalkyl lower alkyl or ZY; wherein R_2 and R_3 may be unsubstituted or substituted with at least one electron withdrawing group or electron donating group , and wherein heterocyclic in R_2 and R_3 is furyl, thienyl, pyrazolyl, pyrrolyl, imidazolyl, indolyl, thiazolyl, oxazolyl, isothiazolyl, isoxazolyl, piperidyl, pyrrolinyl, piperazinyl, quinolyl, triazolyl, tetrazolyl, isoquinolyl, benzofuryl, benzothienyl, morpholinyl, benzoxazolyl, tetrahydrofuryl, pyranyl, indazolyl, purinyl, indolinyl, pyrazolindinyl, imidazolinyl, imidazolindinyl, pyrrolidinyl, furazanyl, N-methylindolyl, methylfuryl, pyridazinyl, pyrimidinyl, pyrazinyl, epoxy, aziridino, oxetanyl or azetidinyl;

Z is O, S, or NR_6';

Y is hydrogen, lower alkyl, aryl, aryl lower alkyl, lower alkenyl, or lower alkynyl, and Y may be unsubstituted or substituted with an electron donating group or an electron withdrawing group, or

ZY taken together is $NR_4NR_5R_7$, NR_4OR_5, or ONR_4R_7;

R_6' is hydrogen or lower alkyl and R_6' may be unsubstituted or substituted with an electron withdrawing group or an electron donating group;

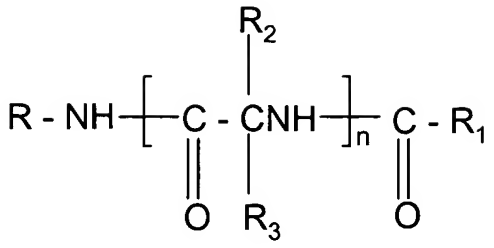
R_4 and R_5 are independently hydrogen, lower alkyl, aryl, aryl lower alkyl, lower alkenyl, or lower alkynyl, wherein R_4 and R_5 are independently unsubstituted or substituted with an electron withdrawing group or an electron donating group; and

R_7 is $COOR_8$, COR_8, hydrogen, lower alkyl, aryl, or aryl lower alkyl wherein R_7 may be unsubstituted or substituted with an electron withdrawing group or electron donating group;

R_8 is hydrogen or lower alkyl, or aryl lower alkyl, and the aryl or alkyl group may be unsubstituted or substituted with an electron withdrawing group or an electron donating group; and

n is 1; wherein the electron withdrawing group and electron donating group are selected from the group consisting of halo, nitro, lower alkenyl, lower alkynyl, formyl, aryl, trifluoromethyl, aryl lower alkanoyl, lower alkoxy carbonyl, hydroxy, lower alkoxy, lower alkyl, mercapto, lower alkylthio and lower alkyldithio.

It is also directed, inter alia, to a method of treating a patient suffering from bipolar disorder comprising administering to said patient a therapeutically amount of a compound of the formula:



wherein

R is aryl lower alkyl and R is unsubstituted or is substituted with at least one electron withdrawing group or electron donating group selected from the group consisting of halo, nitro, lower alkenyl, lower alkynyl, formyl, aryl, trifluoromethyl, lower alkoxy carbonyl, aryl lower alkanoyl, hydroxy, lower alkoxy, lower alkyl, mercapto, lower alkylthio, and lower alkyldithio;

R_1 is methyl, and is unsubstituted or substituted with an electron donating group or an electron withdrawing group selected from the group consisting of halo, nitro, lower alkenyl, lower alkynyl, formyl, aryl, trifluoromethyl, lower alkoxy carbonyl, aryl lower alkanoyl, hydroxy, lower alkoxy, lower alkyl, mercapto, lower alkylthio, and lower alkyldithio;

R_2 is hydrogen, lower alkyl, lower alkenyl, lower alkynyl, aryl, aryl lower alkyl, halo, heterocyclic, heterocyclic lower alkyl, lower alkyl heterocyclic, lower cycloalkyl, lower cycloalkyl lower alkyl, or ZY;

R_3 is lower alkyl, lower alkenyl, lower alkynyl, aryl, aryl lower alkyl, halo, heterocyclic, heterocyclic lower alkyl, lower alkyl heterocyclic, lower cycloalkyl, lower cycloalkyl lower alkyl or ZY;

wherein R_2 and R_3 may be unsubstituted or substituted with at least one electron withdrawing group or electron donating group and wherein heterocyclic in R_2 and R_3 is furyl, thienyl, pyrazolyl, pyrrolyl, imidazolyl, indolyl, thiazolyl, oxazolyl, isothiazolyl, isoxazolyl, piperidyl, pyrrolinyl, piperazinyl, quinolyl, triazolyl, tetrazolyl, isoquinolyl, benzofuryl, benzothienyl, morpholinyl, benzoxazolyl, tetrahydrofuryl, pyranyl, indazolyl, purinyl, indolinyl,

pyrazolindinyl, imidazolinyl, imidazolindinyl, pyrrolidinyl, furazanyl, N-methylindolyl, methylfuryl, pyridazinyl, pyrimidinyl, pyrazinyl, epoxy, aziridino, oxetanyl or azetidinyl;

Z is O, S, or NR_6';

Y is hydrogen, lower alkyl, aryl, aryl lower alkyl, lower alkenyl or lower alkynyl, and Y may be unsubstituted or substituted with an electron donating group or an electron withdrawing group, or

ZY taken together is $NR_4NR_5R_7$, NR_4OR_5, or ONR_4R_7;

R_6' is hydrogen or lower alkyl;

R_4 and R_5 are independently hydrogen, lower alkyl, aryl, aryl lower alkyl, lower alkenyl, or lower alkynyl, and R_4 and R_5 may be independently unsubstituted or substituted with an electron withdrawing group or an electron donating group;

R_7 is $COOR_8$, COR_8, hydrogen, lower alkyl, aryl or aryl lower alkyl, which R_7 may be unsubstituted or substituted with an electron withdrawing group or electron donating group;

R_8 is hydrogen or lower alkyl, or aryl lower alkyl, and the aryl or alkyl group may be unsubstituted or substituted with an electron withdrawing group or an electron donating group; and

n is 1.

5. I have reviewed the Official Action dated September 23, 2005. I have been requested by counsel to submit additional data and to more particularly illustrate that the compounds discussed hereinabove are useful for the treatment of bipolar disorder as described in the instant application.

6. The experiments described in the application and in the present Declaration were conducted under my direct supervision and control. I was fully cognizant of all aspects of the experiments performed and I have interpreted the data as described hereinbelow.

7. The first set of experiments utilizes a representative compound within the scope of the formula described in Paragraph 4. More specifically, (2R)-2-(acetylamino)-N-[(3-fluorophenyl)methyl]-3-methoxypropionamide, a representative compound of the present invention, which is designated as Compound 1 herein, was tested according to the following procedure.

8. The protocol is based on the assay developed by Williams, et al., *Nature*, 417: 292-295 (2002) attached hereto as Exhibit A and subsequently extended by Cheng et al., *Mol. Cell. Neurosci.*, 29: 155-161 (2005) attached hereto as Exhibit B and Di Daniel et al., *Mol. Cell. Neurosci.*, (in press) (2006), attached hereto as Exhibit C to assess both inhibition of growth cone collapse and its reversal in neurons.

9. This assay, identified as the growth cone assay, is based on the common effect on neurons of three widely prescribed drugs (lithium, valproic acid, and carbamazepine) used for the treatment of bipolar disorder. More specifically, as described on Page 5 of the article in Exhibit C, the action of the test drugs which exhibit a positive response, such as the aforementioned anti-convulsants, "on growth cones parallels their clinical efficacy in the treatment of bipolar disorder..." As shown by these experiments, lithium, valproic acid and carbamazepine each increase the spread area of growth cones of neurons derived from the rat cerebral cortex, which effects are reversed by inositol. Furthermore, this assay is consistent with the finding that gabapentin and phenytoin, two other anti-convulsant drugs, which are not

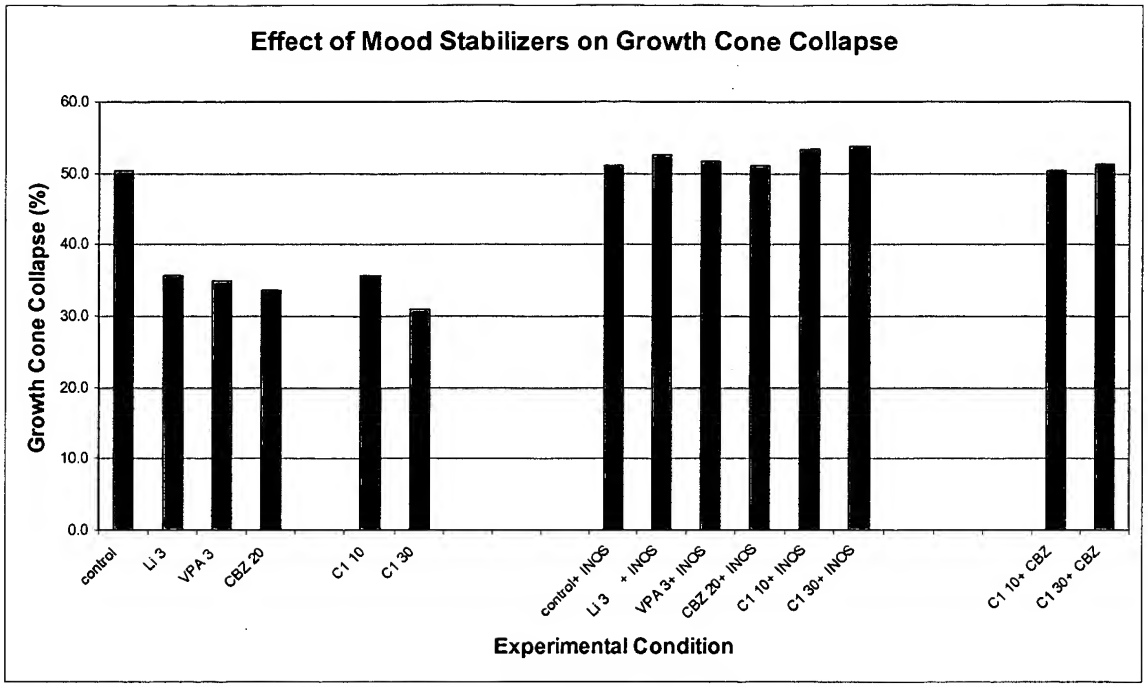
commonly used to treat bipolar disorder, do not mimic the effects of the mood stabilizers, e.g., valproic acid, lithium or carbamazepine, on sensory neuron growth cones.

10. Thus, the assay can differentiate between those drugs which mimic the effects of the mood stabilizers on sensory neuron growth cones and those which do not so mimic. Those that mimic the effect of mood stabilizers on sensory neuron growth cones are indicated to be useful for treating bipolar disorder.

11. The procedure of the assay is as follows: Briefly, dorsal root ganglia from newborn rats were cultured as explants plated on PDL- and laminin-coated coverslips in serum-free medium with nerve growth factor. The compounds tested–Compound 1, lithium, valproic acid (VPA), carbamazepine (CBZ), and vehicle control–were added after approximately 24 hours at the doses indicated below. After approximately 18 hours, the sensory neuron axons that extend from the ganglia were loaded with Calcein and then fixed. The number of collapsed growth cones was counted using a Zeiss fluorescence microscope and the growth cone spread area was calculated.

12. Compound 1 was also tested for reversal of the effect of CBZ on growth cone collapse.

13. The results from both experiments are shown below.



(Concentrations for Compound 1 (C1) and CBZ are expressed as μM; concentrations for lithium and VPA are expressed as mM)

14. As shown by the data, Compound 1 behaved in a similar fashion to the known mood stabilizers lithium and VPA. Compound 1, like lithium, VPA, CBZ, and other drugs effective for the treatment of the manic component of bipolar disorder, inhibited the collapse of the growth cones and this inhibition was reversed by increasing extra-cellular inositol.

15. As displayed in the two bars at the far right of the graph, Compound 1 also reversed the effect of CBZ on growth cone collapse; this effect is similar to that observed in this model with compounds (like lithium and VPA) that also have an effect on the depressant component of bipolar disorder.

16. Thus, the experiments demonstrate that Compound 1 behaves in the present assay like lithium and VPA, which have mood stabilizing activity and affect both components of bipolar disorder [bipolar mania and bipolar depression].

17. A second set of experiments utilized a different representative compound described in the instant specification and in Paragraph 4 hereinabove, viz, 2-(acetylamino)-2-

(methoxyamino)-N-benzylacetamide, which is designated as Compound 2 herein. This second set of experiments is an in vivo assay conducted in mice.

18. The method utilized follows the procedure described by Costall *et al.* in *Brain Research*, 123, 89-111 (1977), the contents of which are incorporated by reference. It uses an activity meter similar to that described by Boissier and Simon in *Arch. Int. Pharmacodyn.*, 158, 212-221 (1965).

19. The procedure of the assay is as follows: Male Rj: NMRI mice (10 per group) were injected with d-amphetamine (3mg/kg i.p.) and were placed immediately in an activity meter. The activity meter consisted of 24 covered plexiglass enclosures (25 x 20 x 9 cm), each equipped with two photocell assemblies contained within a darkened enclosure and connected to silent electronic counters. The number of interruptions by each animal (one per cage) of the photo-electric beams was recorded by computer at 10 minute intervals for 30 minutes. The scores were cumulated over the 30 minute period. The test was performed blind.

Compound 2 was evaluated at 10, 20, and 40 mg/kg administered i.p. 30 minutes before amphetamine administration. Lithium carbonate (4 mEq/kg) was administered under the same experimental conditions, and was used as a comparative agent.

20. The results are tabulated hereinbelow:

TABLE 1

TREATMENT (mg/kg) i.p. -30 min	**TREATMENT (mg/kg) i.p.**	**NUMBER OF PHOTO-BEAMS CROSSED (0 to 30 min)**							
		Mean ± s.e.m.			**p value**	**% change from control**		**% antagonism**
Vehicle (saline)	Vehicle	251.7 ± 17.5						
Vehicle	Amphetamine (3)	630.9 ± 102.8	**	(a)	0.002	+151%	(a)	-
Compound 2 (10)	Vehicle	220.6 ± 19.4	NS	(a)	0.249	-12%	(a)	-
Compound 2 (20)	Vehicle	199.2 ± 30.0	NS	(a)	0.148	-21%	(a)	-
Compound 2 (40)	Vehicle	159.8 ± 19.7	**	(a)	0.003	-37%	(a)	-

Compound 2 (10)	Amphetamine (3)	402.8 ± 54.3	NS	(b)	0.065	-36%	(b)	52%
Compound 2 (20)	Amphetamine (3)	428.3 ± 82.3	NS	(b)	0.141	-32%	(b)	40%
Compound 2 (40)	Amphetamine (3)	173.9 ± 31.3	***	(b)	0.000	-72%	(b)	96%
Lithium (4 mEq/kg)	Vehicle	65.1 ± 10.2	***	(a)	0.000	-74%	(a)	-
Lithium (4 mEq/kg)	Amphetamine (3)	241.4 ± 43.1	**	(b)	0.003	-62%	(b)	54%

NS = Not Significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

(a): compared with control without amphetamine
(b): compared with control with amphetamine

21. The data show that amphetamine (3mg/kg i.p) induced clear hyperactivity (+151 %). In addition, the data show that Compound 2 (10, 20, and 40 mg/kg i.p.), administered alone, moderately decreased locomotion. Furthermore, Compound 2 (10, 20 and 40 mg/kg i.p.), co-administered with amphetamine, tended to antagonize amphetamine–induced hyperactivity at all doses, with the most marked antagonism (96 %) being observed at 40 mg/kg. Lithium (4 mEq/kg i.p.) also decreased locomotion when administered alone and significantly antagonized amphetamine-induced hyperactivity (54 %).

22. Based on the data, it is clear that the Compound 2 behaved similarly to lithium in this assay.

23. Since Compound 2 behaves like lithium, and since lithium is used to treat bipolar disorder, one can conclude that Compound 2 is useful for treating bipolar disorder.

24. The compounds tested in both assays are representative of the compounds described in Paragraph 4 herein.

25. The data clearly illustrate that compounds of the present invention are pharmacologically active in the growth cone assay and in an animal assay model, both of which respond to lithium, a current treatment for bipolar disorder.

26. This data in both sets of experiments clearly support the allegations in the instant application that compounds described in the instant specification and in Paragraph 4 herein are effectively useful for the treatment of bipolar disorder.

27. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

April 12, 2006
Dated

Robert H. Harris, Ph.D.
Robert H. Harris, Ph.D.

A common mechanism of action for three mood-stabilizing drugs

Robin S. B. Williams*, Lili Cheng†, Anne W. Mudge†‡ & Adrian J. Harwood*‡

** Intracellular Signalling and † Cellular Neurobiology Groups, MRC Laboratory for Molecular Cell Biology, and Department of Biology, University College London, Gower St, London WC1E 6BT, UK*
‡ These authors contributed equally to this work

Lithium, carbamazepine and valproic acid are effective mood-stabilizing treatments for bipolar affective disorder. The molecular mechanisms underlying the actions of these drugs and the illness itself are unknown. Berridge and colleagues[1] suggested that inositol depletion may be the way that lithium works in bipolar affective disorder, but others have suggested that glycogen synthase kinase[2,3] (GSK3) may be the relevant target. The action of valproic acid has been linked to both inositol depletion[4,5] and to inhibition of histone deacetylase[6] (HDAC). We show here that all three drugs inhibit the collapse of sensory neuron growth cones and increase growth cone area. These effects do not depend on GSK3 or HDAC inhibition. Inositol, however, reverses the effects of the drugs on growth cones, thus implicating inositol depletion in their action. Moreover, the development of *Dictyostelium* is sensitive to lithium[7] and to valproic acid, but resistance to both is conferred by deletion of the gene that codes for prolyl oligopeptidase, which also regulates inositol metabolism. Inhibitors of prolyl oligopeptidase reverse the effects of all three drugs on sensory neuron growth cone area and collapse. These results suggest a molecular basis for both bipolar affective disorder and its treatment.

In addition to their use as mood stabilizers, carbamazepine (CBZ) and valproic acid (VPA) are also used to treat epilepsy, and VPA[6] and lithium[2] are teratogenic. Their different effects have made it difficult to establish whether the three drugs act on similar cell and molecular targets to stabilize mood in bipolar affective disorder. We reasoned that if it were possible to identify common cellular effects of these structurally diverse mood stabilizers, this might suggest which molecular targets are relevant for their effects on mood and hence the underlying defect in bipolar affective disorder. The cellular effects of lithium have been studied extensively using cultured mammalian neurons as well as lower organisms, but there have been few comparable studies with CBZ and VPA. We therefore reinvestigated the effects of lithium on cultured neurons and compared them with those of CBZ and VPA.

We cultured explants of sensory neurons from newborn rat dorsal root ganglia and tested the drugs at concentrations similar to their recommended therapeutic plasma levels[8]. Consistent with previous studies[3,9,10], lithium treatment resulted in unbundled microtubules in axons, which frequently adopted a zig-zag trajectory (Fig. 1a, left panel). About 2% of growth cones became very large, with microtubules extending abnormally into the growth cones (Fig. 1a, right panel). These effects on microtubule structure were not observed with CBZ or VPA treatment. Lithium also caused a large increase in the number of collateral axon branches that formed proximal to the growth cones (Fig. 1a, b), whereas CBZ had no effect and VPA decreased the number of such branches (Fig. 1b). When lithium and VPA were used together, axons had the appearance of those treated with VPA alone (Fig. 1b).

The HDAC inhibitor tricostatin A (TSA) mimics the teratogenic effects of VPA[6]. TSA also had the same effect as VPA in decreasing the number of collateral axon branches, suggesting that inhibition

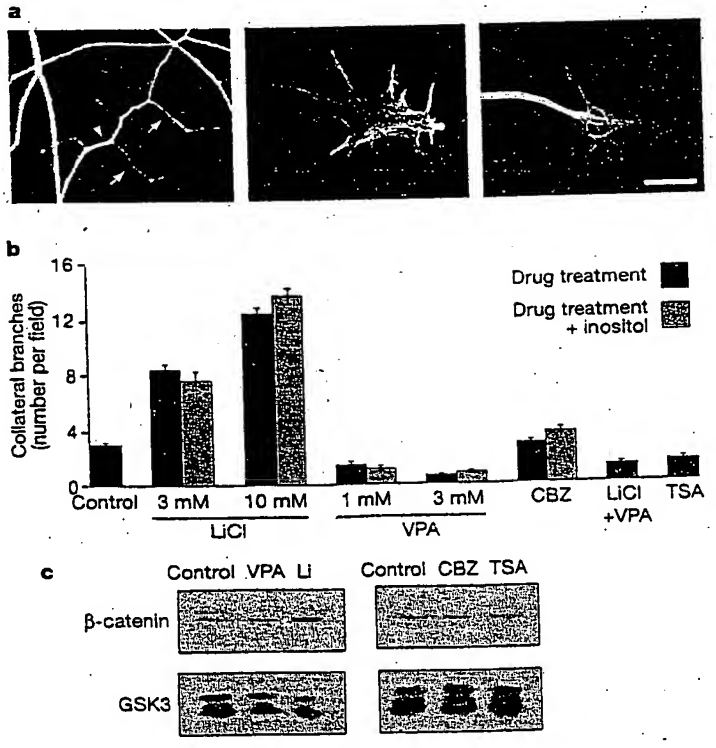


Figure 1 Structural changes in sensory neuron axons following treatment with mood-stabilizing drugs. **a**, Micrograph (left panel) showing axons with a zig-zag trajectory (arrowhead) and collateral branches (arrows) in lithium-treated cultures. Micrographs of an abnormal growth cone double-labelled with phalloidin (middle panel) and anti-acetylated tubulin (right panel), showing coiled microtubules in the growth cone of a lithium-treated axon. Scale bar, 10 μm. **b**, Histograms showing the average number of collateral branches per field after treatment with drugs as indicated (mean ± s.e.m., $n = 60$ fields). **c**, Western blot analysis of β-catenin and GSK3 levels in sensory neurons after drug treatment as indicated. VPA, valproic acid; CBZ, carbamazepine; TSA, tricostatin A.

of axonal branching by VPA depends on the inhibition of HDAC (Fig. 1b). Lithium is thought to alter microtubule and axonal structure by inhibition of GSK3 (ref. 3), and deletion of the genes that code for the microtubule-associated proteins tau or MAP1B—both substrates of GSK3—leads to abnormalities in growth cones similar to those seen with lithium treatment[11]. Lithium, but not CBZ or VPA, inhibits the activity of both the α and β isoforms of GSK3 (ref. 2) (W. J. Ryves and A. J. H., unpublished data). β-catenin is regulated in most cells by GSK3 (refs 12, 13) and by HDAC in a neuroblastoma cell line[6]. Lithium, but not CBZ or VPA, increased β-catenin in our dorsal root ganglia cultures (Fig. 1b). Thus, although both GSK3 and HDAC influence axon growth, their inhibition changes axon morphology in different ways, and they are unlikely to be a common target for lithium, CBZ and VPA.

All three drugs, however, had similar effects on the dynamic behaviour of sensory neuron growth cones. When untreated cultures were viewed by time-lapse video microscopy, the growth cones had pronounced cycles of complete collapse followed by active spreading. Each of the drugs reduced the frequency of collapse and the growth cones were enlarged following each treatment compared with controls (L.C. and A.W.M., unpublished data). The increase in growth cone size may be a consequence of the effect of inhibiting collapse. We quantified these effects by analysing the frequency distribution of collapsed and spread growth cones using fixed and labelled cultures (Fig. 2a, b). In untreated cultures, 27% of growth cones were collapsed (first bar in histograms), whereas CBZ and VPA reduced this proportion five fold, to about 5%, while lithium decreased it 2–3-fold. Lithium, CBZ and VPA also increased the average spread area of growth cones by 56%, 63% and 81%, respectively. These effects on growth cones have not been reported in previous studies with lithium, perhaps because we unmasked the effects by using serum-free medium, where we find that collapse is more pronounced than in serum-containing medium. To our knowledge, this is the first report of a common effect of all three drugs on neurons.

Lithium inhibits inositol monophosphatase (IMPase) and inositol polyphosphatase, thereby leading to a decreased inositol-1,4,5-trisphosphate ($InsP_3$) response[1]. This inhibition prevents both recycling of inositol from inositol phosphates and *de novo* inositol synthesis, and can be overcome by addition of extracellular inositol. The effects of the three drugs on growth cone collapse and area were abolished by addition of inositol, implicating inositol phosphate signalling in this common response (Fig. 2b, right panels). In contrast, addition of inositol had no effect on either the lithium-induced increase in collateral axon branching and giant growth cones, or the VPA-induced decrease in collateral branching (Fig. 1b): this argues against a role for inositol depletion in any of these processes. TSA had no effect on the growth cones, and we showed above that neither CBZ nor VPA affects GSK3: thus, neither HDAC nor GSK3 are likely to be involved in mediating the drug effects on the dynamic behaviour of growth cones.

In all our studies, we observed significant effects at drug concentrations close to the recommended therapeutic plasma levels[8] (0.6–1.0 mM lithium, 0.3–0.6 mM VPA and 20–50 μM CBZ). The levels in clinical use, however, are limited by side effects, and they do not necessarily elicit maximum mood-stabilizing effects. Lithium and VPA are used at remarkably high concentrations: VPA, however, binds to protein and membranes, and the free concentration may be much lower. In the case of lithium, it is notable that the inhibition constant, K_i, for IMPase and GSK3 is 0.8 mM and 2 mM, respectively. Although our studies used growth cones that are characteristic of developing neurons, we think it likely that the effects we describe on inositol depletion will also apply to neurons in the mature brain.

Dictyostelium offers a genetic route to the analysis of drug action. During early development, cells aggregate by chemotaxis to form a multicellular mass. Aggregation is defective when $InsP_3$ signalling is disrupted by lithium, whereas the effects of lithium on later development are due to GSK3 inhibition. Late development is also sensitive to VPA[14], but the mechanism is unknown. We found that VPA reduced basal $InsP_3$ concentration (Fig. 3a). Transcription of the inositol-1-phosphate synthase (*ino1*) gene in yeast is repressed by inositol, and so is an *in vivo* indicator of inositol concentration[5]. As shown in Fig. 3b, 10 mM lithium caused a twofold increase in *ino1* expression, whereas 1 mM VPA produced

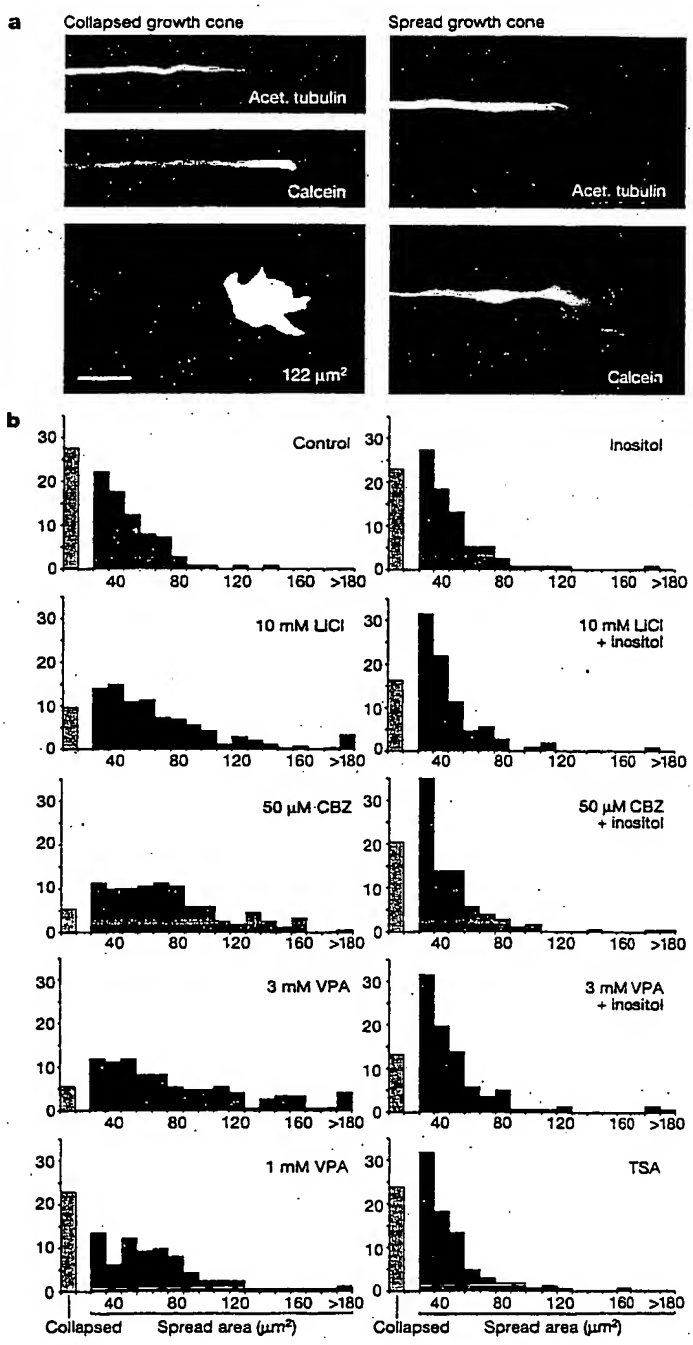


Figure 2 Inositol reverses the effects of mood-stabilizing drugs on growth cone collapse and spread area. a, Collapsed growth cones (top left panels) and spread growth cones (right panels) showing stable microtubules (acetylated tubulin) and cytoplasm (calcein). The area of this growth cone was calculated as 122 μm² as shown (bottom left panel) Scale bar, 10 μm. b, Histograms show the frequency distribution of collapsed growth cones (grey first bar) and the spread area of growth cones plotted in increments of 10 μm² (black bars) expressed as a percentage of the total. The drug treatment for left-hand panels is indicated on each plot. The right-hand panels are from cultures treated with drugs in the presence of 1 mM inositol, or cultures treated with TSA alone as indicated. Data were pooled from up to 5 different platings; the total number of growth cones scored for each treatment was about 150.

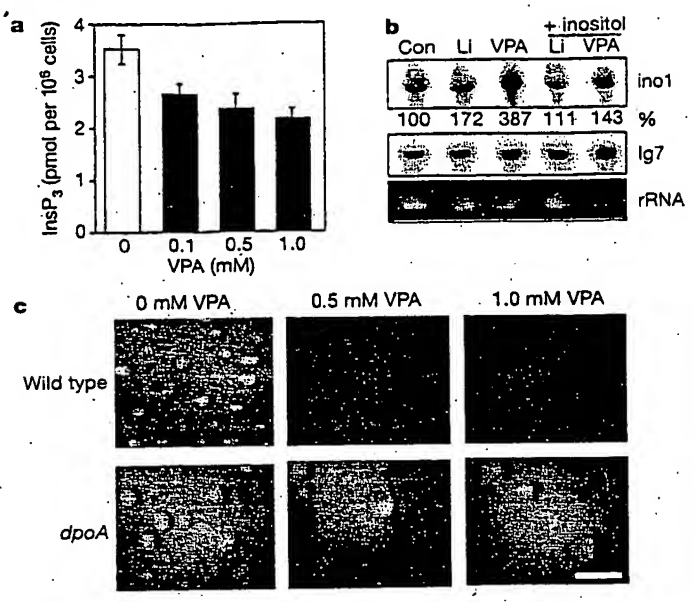


Figure 3 VPA effects on *Dictyostelium* aggregation are due to modulation of $InsP_3$ signalling. **a**, Basal $InsP_3$ levels were measured with and without treatment with VPA. **b**, Expression of the inositol-1-phosphate synthase gene was assessed by northern analysis. Cells were treated with 10 mM lithium, 1 mM VPA, in the absence or presence of 10 mM inositol. Increased *ino1* expression is shown relative to Ig7 (loading control). Ethidium stained rRNA is also shown. **c**, Aggregation of wild-type *Dictyostelium* cells and a mutant lacking the gene that codes for prolyl oligopeptidase (*dpoA*) in 0, 0.5 and 1 mM VPA.

a fourfold increase: both treatments were reversed by inositol addition. This indicates that both drugs reduce the *in vivo* concentration of inositol.

We previously used insertional mutagenesis to generate lithium-resistant *Dictyostelium* mutants[7]. Loss of the gene *dpoA*, which encodes a homologue of the enzyme prolyl oligopeptidase, confers lithium resistance[7] and is associated with elevated basal levels of $InsP_3$. As shown in Fig. 3c, loss of *dpoA* also conferred cross-resistance to VPA, strongly suggesting that $InsP_3$ is a major target of VPA in *Dictyostelium*, and that elevation of basal $InsP_3$ can counteract the VPA effect on aggregation. These direct biochemical measurements and genetic interactions support our conclusion that VPA can target inositol phosphate metabolism in sensory neurons.

Prolyl oligopeptidase is a cytoplasmic protein in both mammals and *Dictyostelium*. It can cleave short oligopeptides at prolyl bonds, but its function is not known. Plasma concentrations of this protein are abnormal in people with affective disorders[15,16]—they are elevated in mania and decreased in depression—although the significance of the finding is unclear. We therefore tested whether there is a link between prolyl oligopeptidase and the action of the mood-stabilizing drugs on neurons. We used two specific inhibitors of prolyl oligopeptidase activity[17,18], and found that both abolished the effects of lithium, CBZ and VPA on growth cone collapse and area. The frequency distributions in Fig. 4 show that there is no significant difference between control growth cones and those treated with drugs in the presence of the inhibitors. In all cases, the number of collapsed growth cones was 26% compared with 27% for untreated controls. The spread area of growth cones in the presence of drugs plus inhibitors was 42 μm^2 compared with untreated controls, which was 43 μm^2. This result suggests that prolyl oligopeptidase may be involved in regulating inositol metabolism in mammalian cells.

Our findings support the hypothesis[1] that the therapeutic target of lithium in the treatment of bipolar affective disorder depends on inositol depletion, and further extends the hypothesis to CBZ and VPA. The brain is highly sensitive to inositol depletion as the blood–brain barrier limits the availability of plasma inositol, making it dependent on inositol recycling and synthesis. The association between prolyl oligopeptidase activity and affective disorders is intriguing and should be explored further, particularly in view of our finding that inhibition of this enzyme mimics the action of added inositol in reversing the drug effects. Finally, these results suggest that the development of both clinical diagnostic tests and new therapies for bipolar affective disorder should focus on inositol phosphate metabolism. □

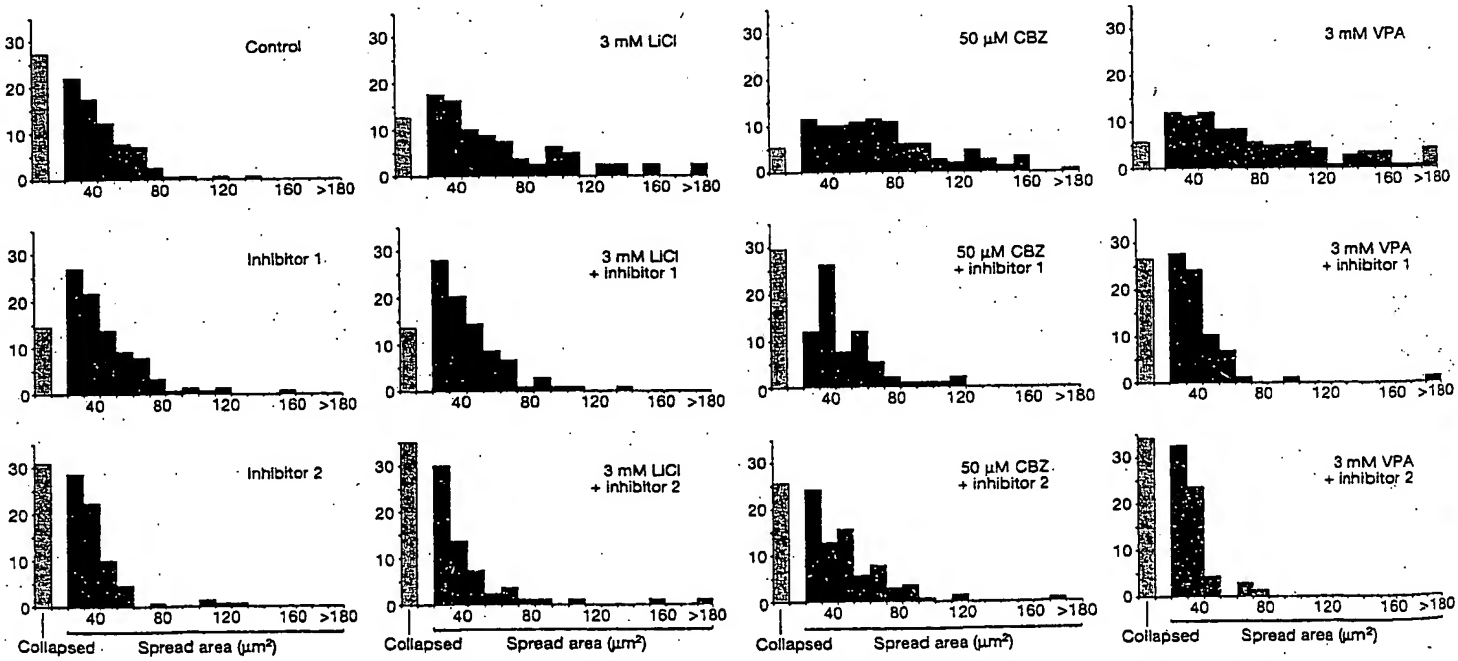


Figure 4 Inhibitors of prolyl oligopeptidase enzyme activity block the action of mood-stabilizing drugs on growth cone collapse and spread area. Histograms show the frequency distribution of collapsed growth cones (grey first bar) and the spread area of growth cones plotted in increments of 10 μm^2 (black bars) expressed as a percentage of the total. The top row shows the effects of the drugs. The second and third rows show the effects of the drugs when they were added in the presence of two structurally unrelated prolyl oligopeptidase inhibitors, which are each specific for the prolyl bond. Inhibitors 1 and 2 are defined in Methods.

Methods

Neuron explant cultures

Dorsal root ganglia from newborn rats were cultured as explants on laminin-coated coverslips in serum-free medium[19] with nerve growth factor (NGF) at 50 μg ml^{-1} (mouse 7S form, Alomone Labs). Cytosine-arabinofuranoside (ara-C, 10 μM) was added after 1 d in culture to kill non-neuronal cells. The drugs to be tested—lithium chloride, 2-propylpentanoic acid (valproic acid, VPA), carbamazepine (CBZ), trichostatin A (TSA), and myo-inositol (all from Sigma)—were also added after 1 d and explants were cultured for a further 2 d before fixation or collection. Trichostatin A was added at 25 μM. Prolyl oligopeptidase inhibitors Z-pro-pro-aldehyde-dimethyl acetal (inhibitor 1, Bachem) and MOC-Glu (NHO-Bz)-Pyr (inhibitor 2, Calbiochem) were added at 133 μM and 24 μM, respectively. Inositol (in the form of myo-inositol) was added at 1 mM.

Immunocytochemistry

Cells were loaded with the fluorescent dye Calcein (Molecular Probes) for 20 min and then fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in cytoskeletal buffer[20] containing 10 mM MES buffer at pH 6.1 with 138 mM KCl, 3 mM $MgCl_2$, and 2 mM EGTA buffer; aldehydes were quenched with sodium borohydride. Cells were then labelled with monoclonal antibody to acetylated tubulin (Sigma, clone 6-11B-1) followed by anti-mouse immunoglobulin, then biotin-streptavidin conjugated to Texas red. In some cases, cells were labelled with fluorescent phalloidin (Alexa^{594}-phalloidin) followed by anti-acetylated tubulin visualized with fluorecein.

Neuron data collection

We scored random fields at the perimeter of the axonal halo that grew out from the explanted cell bodies. Collateral axon branches were counted using acetylated tubulin as the label, and growth cones were scored using the cytoplasmic dye calcein. The growth zone perimeter was traced with a light pen, and the area was computed using OpenLab software. Collapsed growth cones with no calcein-labelled lamellae extending beyond the microtubules were assigned an arbitrary value of 1 for computational purposes. Experiments were done with triplicate coverslips for each treatment, and data were pooled from up to five different platings to generate the histograms shown.

Western blotting

Samples were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Amersham Life Sciences). Western blots were probed with anti-β-catenin[21] (C19220, Transduction Laboratories) or anti-GSK3 (4G-1E, Upstate Biotechnology) primary antibodies. Blots were then labelled with anti-mouse IgG-HRP antibody (Vector Laboratories) and visualized using SuperSignal West Pico Luminol/Enhancer solution (Pierce) according to the supplier's instructions.

Dictyostelium cell culture and development

Wild-type *Dictyostelium discoideum* cells (Ax2) or *dpoA* mutant cells[7] were grown at 22 °C in shaking culture in HL5 medium to 3×10^6 cells ml^{-1} or in association with *Klebsiella aerogenes*. Effects of drug treatment on aggregation was analysed by plating cells at 5×10^6 per 47 mm nitrocellulose filter (Millipore) soaked in KK_2 (16.5 mM KH_2PO_4, 8 mM K_2HPO_4 pH 6.2) containing lithium or VPA (buffered to pH 7.0) as indicated. Development was observed after 8 h.

$InsP_3$ assays

Wild-type cells were grown in shaking suspension for 20 h at 2×10^6 cells ml^{-1} containing defined concentrations of VPA. Cells were re-suspended at 5×10^7 cells ml^{-1} and aerated for 10 min. Cells (200 μl) were lysed and the $InsP_3$ concentrations measured by an isotope dilution binding assay (Amersham Pharmacia Biotech).

Northern analysis

Cells were treated with VPA or LiCl as described for $InsP_3$ assays, and RNA was prepared as previously described[22]. The *ino1* cDNA (inositol-1-phosphate synthase, clone SLB 678), provided by the *Dictyostelium* cDNA project in Japan, was labelled by random priming, and hybridized at 40 °C in hybridization solution containing 42% formamide[23].

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Competing interests statement

The authors declare that they have no competing financial interests.

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The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in *Xenopus* embryos

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It is thought that inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$)-Ca^{2+} signalling has a function in dorsoventral axis formation in *Xenopus* embryos[1–3]; however, the immediate target of free Ca^{2+} is unclear. The secreted Wnt protein family comprises two functional groups, the canonical Wnt and Wnt/Ca^{2+} pathways[4]. The Wnt/Ca^{2+} pathway interferes with the canonical Wnt path-

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How can the mood stabilizer VPA limit both mania and depression?

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The mood stabilizing drugs commonly used to treat bipolar disorder–lithium, valproic acid (VPA), and carbamazepine (CBZ)–limit the frequency of swings to either manic or depressive states. We previously showed that these drugs all have a common action on cultured neurons, which can be reversed by the addition of either inositol or specific inhibitors of the enzyme prolyl oligopeptidase (PO). Inhibition of PO activity is reported to enhance phosphoinositide (PIns) signaling consistent with the suggestion that mood stabilizers inhibit PIns signaling. We now report that VPA directly inhibits recombinant PO activity, which would have the opposite effect on PIns signaling. This unexpected result suggests a model that could explain the dual action of VPA in stabilizing mood: We propose that euthymic mood is dependent on stable PIns signaling and that VPA may limit mood swings to mania by decreasing PIns signaling, and that it may limit mood swings to depression by inhibiting PO and thus increasing PIns signaling.

Introduction

Bipolar disorder is ranked by the World Health Organisation as the sixth leading cause of disability worldwide. It is characterized by severe mood swings to both mania (Type 1) or hypomania (Type 2) and depression. The mood-stabilizing drugs commonly used to treat this illness are often very effective in controlling symptoms, but in many cases intolerable side-effects make them unacceptable. The therapeutically relevant molecular targets of the mood-stabilizing drugs are not known. Identification of them should provide specific targets for the development of more effective and specific drugs with fewer side-effects: In addition, understanding how the drugs work should shed light on the underlying causes of bipolar disorder (Gould et al., 2004; Williams et al., 2002). Several lines of evidence suggest that neuronal PIns signaling plays a key role in mood control (Agam et al., 2002; Berridge et al., 1989; O'Donnell et al., 2000; Silverstone et al., 2002; van Calker and Belmaker, 2000; Williams et al., 2002). We showed that the three mood stabilizers–lithium, VPA, and CBZ–each decrease the collapse and increase the spread area of growth cones of cultured neurons: inositol addition reversed the drug effects suggesting that each drug caused inositol depletion and/or inhibit PIns signalling (Williams et al., 2002). Inhibitors of the enzyme PO also reversed the effects of the mood stabilizers (see below). To date, the PIns signaling pathway is the only one shown to be targeted by all three mood stabilizers (van Calker and Belmaker, 2000; Williams et al., 2002). Lithium is known to directly inhibit the enzymes that recycle inositol from inositol phosphates (InsPs)–inositol monophosphatase (IMPase) and inositol polyphosphatase (IPPase) (Berridge et al., 1989; Gould et al., 2004)–and VPA indirectly inhibits the enzyme responsible for InsP synthesis—myo-inositol phosphate synthase (MIP-synthase) in yeast (Agam et al., 2002; Ju et al., 2004): the mechanism for inositol depletion by CBZ (Williams et al., 2002) is unknown, but CBZ is reported to inhibit the cyclic adenosine monophosphate (cAMP) second messenger system (Gould et al., 2004). Lithium also inhibits glycogen synthase kinase 3 (GSK3) (Gould et al., 2004; Klein and Melton, 1996), and VPA also inhibits histone deacetylase (HDAC) (Gould et al., 2004; Phiel et al., 2001): these and other signaling pathways affected directly or indirectly by the mood stabilizers (Gould et al., 2004) are not common to all, however, and so seem less likely to be crucial for the control of mania, although they probably contribute to other drug effects including neuroprotection and structural changes in the brain. These alternate drug targets have been discussed in several recent reviews (Coyle and Duman, 2003; Gurvich and Klein, 2002; Harwood and Agam, 2003; Klein and Melton, 1996).

VPA, lithium, and CBZ are all effective antimanic drugs, with only limited efficacy for acute bipolar depression (Goodwin, 2003). The defining (and puzzling) characteristics of the mood stabilizing drugs, however, are that they limit the frequency of mood swings to mania without affecting normal mood (euthymia) and both lithium and VPA can also limit mood swings to depression (Goodwin, 2003). In addition, antipsychotic drugs such as olanzapine are used to control acute mania and recently the anticonvulsant drug lamotrigine was shown to be effective in

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controlling bipolar depression (Calabrese et al., 2002; Goodwin, 2003). The question of how mood stabilizers such as lithium and valproate might function to control both poles of mood is rarely discussed, however, when considering the relevant therapeutic targets for the drugs. In this study, we address the issue of how VPA might limit swings to both mania and depression by controlling both the highs and the lows of PIns signaling via a mechanism involving the enzyme PO.

PO is a serine protease that belongs to a family of structurally-related proteases (Polgar, 2002). PO cleaves prolyl bonds of small (<3 kDa) peptides, whereas the prolyl bonds of proteins are not cleaved because the β-propeller structure of PO restricts access of larger peptides or protein to the active site (Fulop et al., 1998). This preference for small peptides initially focussed attention on neuropeptides as the natural substrates for PO, but because PO is a cytoplasmic enzyme, neuropeptides are unlikely to be substrates because they are not free in the cytoplasm. The intracellular substrate for PO is still not known, but there is evidence suggesting that PO may inhibit the PIns signaling pathway, but again, the mechanism is not known: Deletion of the PO gene in the slime-mold *Dictyostelium* (Williams et al., 1999) and inhibition of PO activity in an astrocytoma cell line (Schulz et al., 2002) results in increased intracellular basal levels of inositol 1, 4, 5-trisphosphate ($InsP_3$) (~2-fold) together with an enhanced response to agonists that stimulate PIns signaling (Schulz et al., 2002). The ability of PO inhibitors to mimic the effect of inositol on the growth cones is consistent with PO being a negative regulator of the PIns signaling pathway in neurons (Williams et al., 2002) and it raised the question of what part PO plays in drug action and mood control. PO is widely distributed in the body but the highest concentration is in the frontal cortex where there are both soluble and membrane-bound forms: the latter are enriched in synaptic structures (Irazusta et al., 2002; O'Leary et al., 1996). PO had been linked previously to mood and stress-related disorders because blood levels of PO in these illnesses differ from levels in control subjects (Maes et al., 1995, 1998), although the significance of these blood studies is still unclear. In addition, PO inhibitors have been tested in Phase 1 trials for their ability to enhance cognition in elderly subjects (Morain et al., 2000). In the present study, we tested whether mood stabilizers or other psychoactive drugs can directly affect PO activity. We find that VPA directly inhibits recombinant PO activity with a K_i of ~1 mM, which is close to the therapeutic blood levels of 0.3–0.7 mM. This result, together with our previous work with mood stabilizing drugs, suggests a model for the ability of VPA to limit swings to both mania and depression.

Results

We first expressed recombinant human PO (rhPO) in bacteria, purified it using a histidine tag, and tested enzyme activity using the synthetic PO substrate Z-Gly-Pro-AMC. As shown in Fig. 1a, VPA inhibited the activity of rhPO with an IC_{50} of 1–2 mM. In contrast, several other psychoactive drugs tested did not significantly affect PO activity at doses consistent with their therapeutic blood levels (10–50 μM): the inactive drugs included the mood stabilizers, lithium and CBZ, the antidepressants, desipramine and fluoxetine, and the antipsychotic, olanzapine (Figs. 1a, b).

To characterize the inhibition of PO by VPA, we performed a detailed analysis of the Michaelis–Menten kinetic parameters using

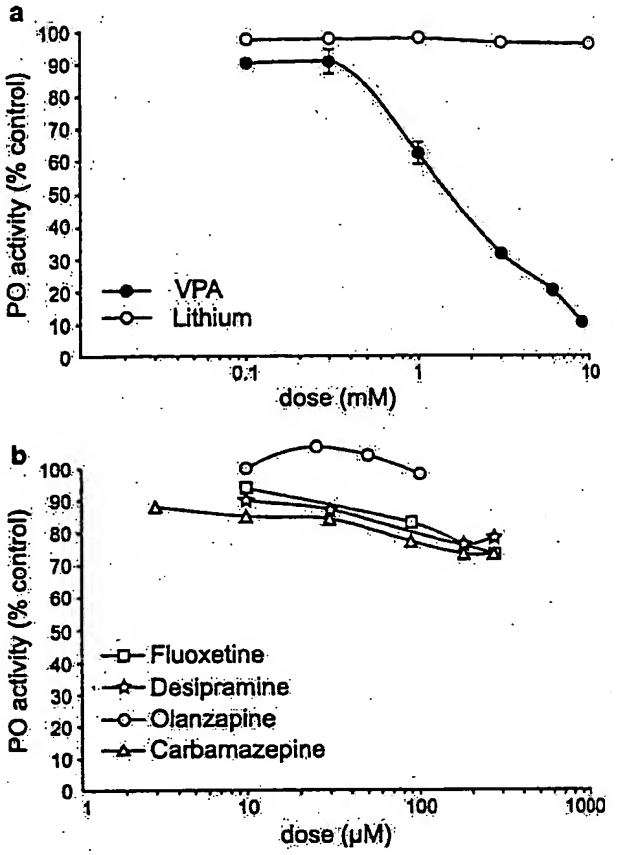
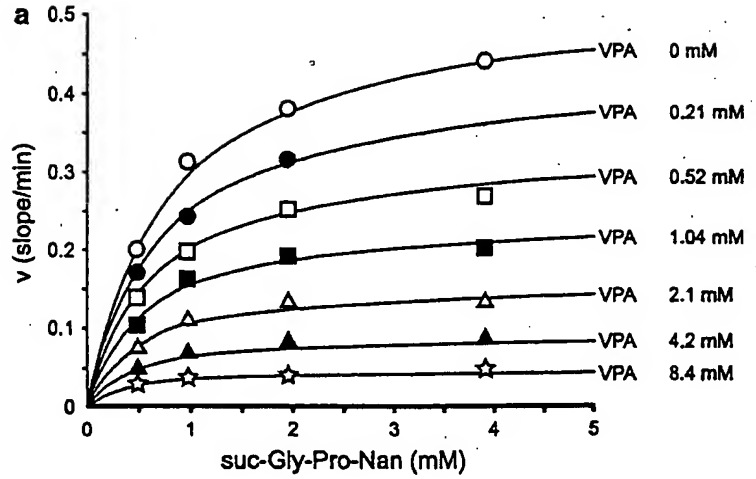
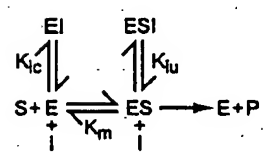


Fig. 1. (a) VPA inhibition of rhPO. PO activity was measured using Z-Gly-Pro-AMC substrate at 30 μM (K_m). Relative activity of PO in the presence of VPA is shown as a percent of activity in the absence of VPA. Lithium tested in the range of 0.1–10 mM had no significant effect on PO activity. (b) Relative activity of rhPO with the drugs CBZ, desipramine, fluoxetine, and olanzapine. PO activity was measured as in (a) but in a lower concentration range (μM) and activity was compared with controls with a matched concentration of DMSO.

pure recombinant porcine PO (rpPO) with two synthetic substrates, Suc-Gly-Pro-Nan (Fig. 2a) or Z-Gly-Pro-Nap (data not shown), as previously described: these substrates show differences in their pH dependence. For both substrates, VPA decreased V_{max} in the dose range 0.2–8 mM (see Fig. 2a): note that there is substantial inhibition of PO activity at 0.5 mM VPA, which is within the standard range for therapeutic blood levels, namely 0.3–0.7 mM. When fitted with the equation describing pure non-competitive inhibition, we obtained a K_i of ~1 mM for both substrates. We obtained better fits with the equation describing mixed inhibition, however, indicating that the inhibition possessed a significant competitive component with both substrates (Figs. 2b, c). This analysis implies that VPA interferes with both the catalytic activity of the enzyme and the binding of substrate to the active site. The inhibition of PO by VPA was specific for this enzyme because VPA, even at a dose of 12 mM, did not inhibit oligopeptidase B (Fig. 3), which cleaves dibasic residues and also belongs to the PO family of serine peptidases—all of which are structurally and mechanistically related (Polgar, 2002). VPA's specificity for PO over oligopeptidase B, together with the competitive inhibitory component, suggests that VPA binding may specifically affect the proline-binding site of PO, which would provide an explanation of





c

	A	A + 150 mM KCl	B + 150 mM KCl
K_m (mM)	0.77 ± 0.05	1.14 ± 0.05	0.011 ± 0.002
K_{ic} (mM)	1.81 ± 0.43	1.51 ± 0.20	2.7 ± 0.7
K_{iu} (mM)	0.87 ± 0.06	0.92 ± 0.07	0.7 ± 0.1

A: substrate Suc-Gly-Pro-Nan
B: substrate Z-Gly-Pro-Nap

Fig. 2. VPA inhibition of rpPO. (a) Initial rates (v) of enzyme activity were monitored versus substrate Suc-Gly-Pro-Nan and VPA concentrations as shown. Results were analyzed using the multidimensional equation for mixed inhibition: $v = V_{max}S / [K_m(1 + I/K_{ic}) + (1 + I/K_{iu})\ S]$ where I (VPA) is the second independent variable and K_{ic} and K_{iu} are the competitive and uncompetitive components of the dissociation constant, V_{max} and K_m are the Michaelis–Menten parameters. The data were fitted by nonlinear regression. (b) Mechanism for mixed inhibition. (c) Table shows the calculated values for K_m, K_{ic} and K_{iu} with either Suc-Gly-Pro-Nan (A) or Z-Gly-Pro-Nap (B) as substrate, in the presence or absence of 150 mM KCl as indicated.

why a short, branched-chain fatty acid has specificity as a drug. Analysis of the structure of PO crystallized with VPA bound should reveal the precise location of VPA binding {see Fulop et al. (1998)}.

The direct inhibition of PO by VPA was at first surprising: because PO inhibitors reversed the action of VPA on growth cones (Williams et al., 2002), we had predicted that if VPA affected PO activity either directly or indirectly, then the effect would be to activate PO rather than inhibit it. We therefore tested whether we could find conditions where the mood stabilizers could have opposite effects on the behavior of the neurons. Indeed, we found that lithium, CBZ, and VPA can have opposite effects on the morphology of neuronal growth cones, depending on the levels of intracellular c-AMP (see below). We tested cAMP effects for three reasons: first, the cyclic-AMP-response-element-binding protein (CREB) is thought to play a major role in depression (Nestler et al., 2002) and is regulated by $InsP_3$ signaling as well as by cAMP (Lonze and Ginty, 2002), suggesting that both these second messengers may be crucial for mood control. Second, cAMP modulates synaptic transmission (Nagy et al., 2004), as does $InsP_3$ (Nagase et al., 2003; Nahorski et al., 2003). Third, a rise in intracellular cAMP switches the response of growth cones to the signal protein semaphorin from attractive to repellent (Nishiyama et al., 2003).

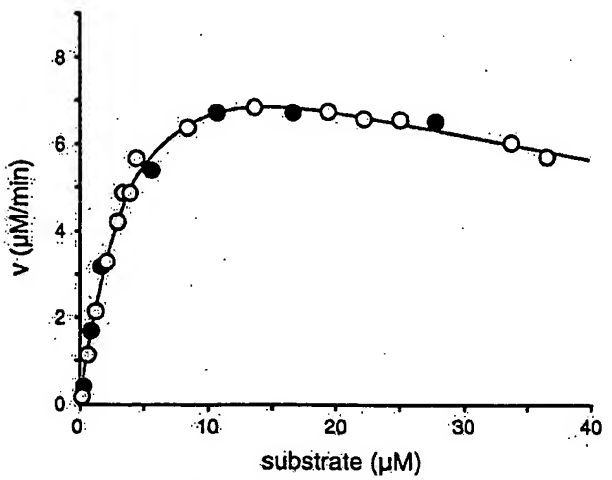


Fig. 3. Oligopeptidase B is not inhibited by VPA. Initial rates (v) were monitored using variable concentrations of the substrate Abz-Thr-Arg-Arg-|-Phe(NO_2)-Ser-Leu-NH_2 as shown. This substrate binds in an alternative unreactive mode, so that the data were fitted by nonlinear regression with a modified Michaelis–Menten equation, $v = V\,[S] / (K_m + [S] + [S]^2/K_{is})$ where K_{is} is the inhibition constant for the second substrate. The filled points were determined in the presence of 12 mM VPA.

We treated cultured sensory neurons with a non-hydrolyzable analogue of cAMP, cpt-cAMP, in order to increase the levels of cAMP-dependent protein phosphorylation. Cpt-cAMP had the same effect as the mood stabilizers on the dynamic behavior of the growth cones (Williams et al., 2002), decreasing the number of collapsed growth cones as previously reported (Fig. 4). When we added the mood stabilizers together with cpt-cAMP, however, the effects were not additive-instead lithium, VPA and CBZ had the opposite effect and reversed the cpt-cAMP-induced collapse. Importantly, the PO inhibitor also reversed the effect of cpt-cAMP on growth cones, consistent with the possibility that VPA reversed the effects of cpt-cAMP on growth cones by inhibiting PO activity. Because cpt-cAMP changed the neuronal response to lithium and CBZ (Fig. 4), but these drugs did not inhibit PO activity directly, it may be that there are additional regulators of PIns signaling that are

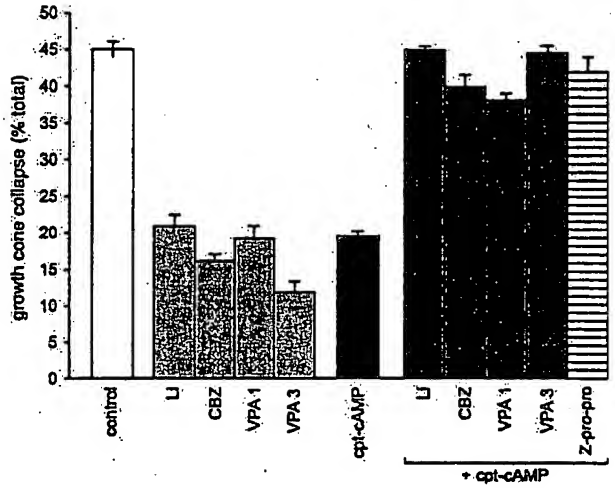


Fig. 4. Cpt-cAMP changes the response of growth cones to mood stabilizers. Sensory neuron explants were treated with 3 mM lithium, 3 mM VPA, 20 μM CBZ, or the PO inhibitor Z-Pro-Prolinal (133 μM) in the presence or absence of 200 μM cpt-cAMP as indicated. The number of collapsed growth cones was counted as a percent of total ($n = 6$).

drug-sensitive and that result in increased PIns signaling as does VPA inhibition of PO.

Discussion

We report that the proteolytic enzyme PO is a direct molecular target for the mood-stabilizing drug, VPA. The K_i for direct inhibition of PO by VPA is ~1.0 mM, which is compatible with the surprisingly high therapeutic blood level of 0.3–0.7 mM that is routinely used to treat bipolar disorder (Taylor et al., 2001). Moreover, VPA accumulates inside cells and it may also be more effective in inhibiting PO action on its native but unknown substrate. (For comparison, the IC_{50} for VPA inhibition of HDAC is 0.4 mM and the K_is for lithium inhibition of IMPase and GSK3 are 0.8 mM and 2 mM, respectively.) Because PO is involved in some way in the regulation of the PIns signaling pathway (Schulz et al., 2002; Williams et al., 1999), this result adds further support to the suggestion that the PIns signaling pathway is involved in the therapeutic action of the mood stabilizing drugs and that defects in the mechanisms that regulate PIns signaling may underlie bipolar disorder. This result also suggests that other small molecule inhibitors of PO may be useful in the treatment of bipolar disorder.

Direct inhibition of PO by VPA was surprising for three reasons: First, we found previously that both PO inhibitors and inositol reverse the effects of VPA and other mood stabilizers indicating that VPA depletes inositol or inhibits the PIns signaling pathway (Williams et al., 2002). Second, VPA lowers inositol levels in the brain (O'Donnell et al., 2000; Silverstone et al., 2002). Third, inhibition of PO activity increases basal $InsP_3$ in astrocytomas (Schulz et al., 2002). We had predicted therefore that VPA might activate PO rather than inhibit it. The unexpected direct inhibition of PO by VPA suggests that, in some circumstances, VPA could increase PIns signaling and, perhaps, inositol availability, the opposite of the effects described previously (Agam et al., 2002; O'Donnell et al., 2000; Williams et al., 2002). Although surprising, our results again point to the PIns signaling pathway as being key for mood control. More importantly, this unexpected effect of VPA suggests an explanation for the dual function of VPA in limiting both depression and mania. Moreover, our experiment showing that the neuronal response to VPA varies with the level of cAMP-dependent phosphorylation in the neurons also suggests that ongoing activity in the brain may alter the response to the drugs, thus allowing either the positive or negative regulation of PIns signaling to predominate, but this suggestion needs further exploration.

The efficacy of drugs in the control of mood seems likely to involve drug regulation of synaptic transmission in relevant brain circuits. Although the PIns signaling pathway is not required for basal synaptic transmission, the intracellular second messengers $InsP_3$ and diacylglycerol (DAG) enhance synaptic transmission and are required for some types of synaptic plasticity (Brose and Rosenmund, 2002; Nahorski et al., 2003), as is the second messenger, cAMP (Nagy et al., 2004). The second messengers can have both acute and long-term effects mediated by changes in gene expression (Lonze and Ginty, 2002). Moreover, given its role in synaptic plasticity, PIns metabolism is likely to be under stringent local control in specific synapses. Both lithium and VPA can decrease inositol levels in brain (O'Donnell et al., 2000; Silverstone et al., 2002), although such global changes are difficult to interpret because PIns metabolism differs between cell types, with glial cells having higher levels than neurons (van Calker and Belmaker, 2000). In addition, PIns signaling is spatially restricted within single neurons (Delmas et al., 2004; Nahorski et al., 2003).

Inositol levels in the brains of manic subjects are reported to be higher than in controls, although the studies so far are limited in subject numbers (Davanzo et al., 2001; Silverstone et al., 2002). In contrast several studies have reported that brain inositol levels are low in depression (Barkai et al., 1978; Frey et al., 1998; Shimon et al., 1997). Interestingly, euthymic subjects treated with either lithium or VPA have normal inositol levels compared with controls, suggesting that the mood stabilizers may return inositol levels and PIns signaling to an optimum compatible with normal mood (Silverstone et al., 2002) (see Fig. 5). In addition, raising the levels of brain inositol improves the mood of depressed subjects in pilot studies (Chengappa et al., 2000; Levine et al., 1995), and inositol acts like an antidepressant in animal studies (Einat and Belmaker, 2001; Einat et al., 1999). These observations, together with our results showing the possibility of dual effects of VPA on PIns signaling, suggest a model for the dual action of this mood stabilizer (see Fig. 5). They also suggest that both the manic and the depressive phases of bipolar disorder may involve defects in the PIns signaling pathway.

In proposing the 'inositol depletion' hypothesis for the action of lithium in the control of mood, Berridge et al. (1989) likened the action of lithium to that of a car seat belt where the restraining force only comes into play when there is pressure on the belt. This model, however, only accounts for euthymia and the control of mood in one direction, most likely mania. We propose instead that the control of mood is more like the action of a sound compressor, which limits extremes by attenuating high and amplifying low volumes to keep music at an optimal level. By analogy with such a sound compressor, the inhibitory effects of the three mood stabilizers on PIns signaling that we described previously (Williams et al., 2002) may act to limit the highs, while the direct inhibition of PO by VPA may act to limit the lows of PIns signaling, thereby maintaining PIns signaling in mood-related circuits within the range required for mood stability. (It is also possible that there are other targets of lithium–not PO–that oppose

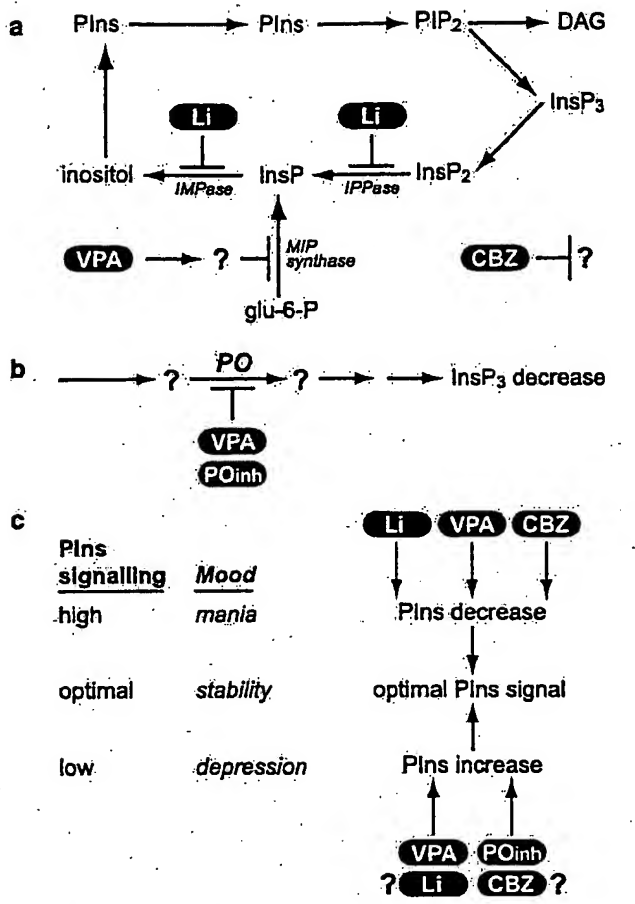


Fig. 5. (a) Diagram showing enzymes in the PIns signaling pathway. The second messengers $InsP_3$ and DAG are generated from phosphatidylinositol 4,5-bisphosphate (PIP_2) and cytoplasmic inositol phosphates are recycled back to phosphoinositides (PIns, PIP and PIP_2) in the membrane. Enzymes inhibited by lithium (Berridge et al., 1989) and possibly VPA (Agam et al., 2002) are indicated by the bars. (b) PO decreases PIns signaling in cells by an unknown mechanism (Schulz et al., 2002; Williams et al., 1999). (c) Model for how PIns signaling defects could be involved in both mania and depression and how VPA and other mood stabilizers may dampen swings in PIns signaling and thus stabilize mood in bipolar disorder.

its PIns inhibitory effects and that could explain lithium's ability to limit depression as we suggest for VPA's inhibition of PO).

In summary, our finding of this new molecular target for VPA, together with our previous study (Williams et al., 2002), strengthens the hypothesis that bipolar disorder involves defects in a regulatory system that acts to dampen swings to either high or low PIns signaling. They suggest that searches for new therapies for bipolar disorder would do well to focus on control elements in the PIns signaling pathway, including PO, which may prove to be a target for drugs that limit the depressive phase of this devastating illness.

Experimental methods

Recombinant PO

To produce recombinant human PO (hrPO) protein, we obtained an IMAGE clone containing the human PO gene (CM276) from HGMP, Hinxton Hall, Cambridge, UK. The coding region was PCR'd and cloned into the bacterial expression vector pTrcHis2 (Invitrogen). The C-terminally His-tagged protein was expressed and purified using the QiaExpress purification system (Qiagen). Recombinant porcine PO rpPO) and oligopeptidase B were prepared and assayed as described previously (Juhasz et al., 2002; Szeltner et al., 2000).

PO enzyme activity assays

All synthetic substrates were from Bachem, Switzerland. Enzyme assays with brain extracts and rhuPO were done in 150 mM potassium phosphate buffer, pH 7.2 at 37°C with the substrate benzyloxycarbonyl-Gly-Pro-7-amido-4-methylcoumarin (Z-Gly-Pro-AMC) (O'Leary et al., 1996). Enzyme activity with rpPO and the substrate succinyl-Gly-Pro-4-nitroanilide (Suc-Gly-Pro-Nan) was measured at 25°C in 75 mM Mes-Tris buffer, pH 7.0, using 19.5 nM enzyme and VPA in the range of 0.2–8mM; in some experiments 150 mM KCl was added to the buffer to mimic the intracellular salt composition. For activity studies with the substrate Z-Gly-Pro-2-naphthylamide (Z-Gly-Pro-Nap), 0.42 nM enzyme was used in buffer with 0.125% acetonitrile and 10 nM BSA. Initial rates (v) were monitored with either Suc-Gly-Pro-Nan or Z-Gly-Pro-Nap in the absence or presence of the inhibitor, VPA. Results were analyzed using the multidimensional equation for mixed inhibition: $v = V_{max}S/[K_m(1 + I/K_{ic}) + (1 + I/K_{iu})S]$, where I (VPA) is the second independent variable and K_{ic} and K_{iu} are the competitive and uncompetitive components of the dissociation constant, V_{max} and K_m are the Michaelis–Menten parameters. The data were fitted by nonlinear regression using the *GraFit* software (Erythacus Software, Ltd., Staines, UK).

Oligopeptidase B enzyme activity assays

The internally quenched fluorescence substrate Abz-Thr-Arg-Arg-|-Phe(NO_2)-Ser-Leu-NH_2 was prepared by solid phase synthesis. The reactions were monitored fluorometrically at 25°C using 0.19 nM enzyme and excitation and emission wavelengths of 337 nm and 420 nm, respectively, as previously described (Juhasz et al., 2002).

Drugs

Stock solutions of drugs were prepared as follows: lithium, VPA and the c-AMP analogue chlorophenylthio-cAMP (cpt-cAMP) were dissolved in water, while CBZ, desipramine, fluoxetine, and olanzapine were each dissolved in DMSO; solvent controls were used appropriately. Drugs were purchased from the following companies: lithium, VPA, and CBZ (Sigma), fluoxetine (Tocris), cpt-cAMP (Calbiochem), Z-Pro-Pro-aldehyde-dimethyl acetal (Z-Pro-Prolinal, Bachem). Olanzapine was generously provided by GlaxoSmithKline (Harlow, UK).

Growth cone assay

Newborn rat dorsal root ganglia were plated on laminin-coated coverslips and cultured in DMEM/F12 medium with the addition of defined additives (Cheng and Mudge, 1996). Cytosine arabinoside (5×10^{-6}M) was added to kill dividing non-neuronal cells and 50 μg ml^{-1} of NGF (7S-form; Alomone Laboratories) was added to promote neuron survival and axonal

outgrowth. Neurons were grown for 24 h in a 5% CO_2 incubator at 37°C before the addition of drugs. After a further 18 h, the sensory neuron axons that extend from the ganglia were loaded with the cytoplasmic dye Calcein (Molecular Probes) and fixed as described previously (Williams et al., 2002). The number of collapsed growth cones was counted using a Zeiss fluorescence microscope and expressed as a percentage of total growth cones: at least 300 growth cones were counted in duplicate in each experiment and the experiment was repeated three times with similar results. Time-lapse movies showing the dynamic behavior of the axonal growth cones in the presence or absence of mood stabilizers or PO inhibitors can be viewed on http://www.ucl.ac.uk/lmcb//anne/Mudge/MechanismOfMoodStabilizers.html.

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The common inositol-reversible effect of mood stabilizers on neurons does not involve GSK3 inhibition, *myo*-inositol-1-phosphate synthase or the sodium-dependent *myo*-inositol transporters

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We previously showed that the mood stabilizers lithium, valproate (VPA), and carbamazepine (CBZ) have a common, inositol-reversible effect on the dynamic behavior of sensory neurons, suggesting that they all inhibit phosphoinositide (PIns) synthesis. We now report similar effects of the drugs in cortical neurons and show by mRNA analysis that these neurons do not express *myo*-inositol-1-phosphate synthase (MIP-synthase) or the sodium-dependent *myo*-inositol transporters (SMIT1 and SMIT2), but they do express the H^+/*myo*-inositol transporter (HMIT) mRNA and protein. We used glycogen synthase kinase-3 (GSK3) inhibitors and Western blotting of GSK3 targets to confirm that the common effects of the drugs on both sensory and cortical neuron growth cones are inositol-dependent and GSK3-independent. Moreover, the anti-convulsant drugs gabapentin and phenytoin do not mimic the mood stabilizers. These results confirm that the common inositol-reversible effect of mood stabilizers on neurons does not involve GSK3 and further show that the effects are independent of MIP-synthase and SMIT transporters.

Keywords: Sensory neurons; GSK3; Phosphoinositide signaling; Lithium; Valproate; Carbamazepine; Inositol; Transporters; MIP-synthase

Introduction

Bipolar disorder is a devastating psychiatric illness that affects about 1–2% of the world population. The mood-stabilizing drugs used to treat this illness target several intracellular signaling pathways and a better understanding of which targets are therapeutically relevant should shed light on the underlying causes of this illness and may also help in the development of more specific drugs. We showed previously that the three most often prescribed mood stabilizers—lithium, VPA, and CBZ—have a common effect on the dynamic behavior of rat sensory neuron growth cones. All three drugs inhibit the collapse and increase the spread area of growth cones (Williams et al., 2002). Moreover, both these effects are reversed by the addition of inositol to the culture medium, suggesting that all three mood-stabilizing drugs inhibit recycling of PIns, because this is the only known effect of inositol on intracellular signaling (Batty and Downes, 1995). Given the commonality of the drug effects in PIns signaling, we hypothesized that this pathway was the most likely therapeutic target for the drugs, and further, that defects in the regulation of PIns signaling may underlie bipolar disorder.

Lithium directly inhibits two key enzymes involved in PIns recycling, inositol monophosphatase (IMPase), and inositol polyphosphate 1-phosphatase (IPPase). Berridge et al. (1989), and later Batty and Downes (1995), suggested that these inhibitory effects on the PIns cycle may partly explain the therapeutic action of lithium. We recently showed that VPA directly inhibits the enzyme prolyl oligopeptidase (PO) (Cheng et al., 2005), which is also implicated in regulation of PIns metabolism, and we proposed that VPA inhibition of PO may partly explain the dual action of the drug in limiting mood swings to both mania and depression (Cheng et al., 2005). Both lithium and VPA treatment decrease brain inositol levels (by ~40% or less) in both rodents and humans (Silverstone et al., 2005), but it is not clear if this global decrease in inositol is relevant for their therapeutic action. Brain inositol levels are regulated by both transport from blood and by synthesis from glucose-6-phosphate to *myo*-inositol-1-phosphate by the enzyme MIP-synthase, whose activity is inhibited indirectly by VPA (Agam et al., 2002; Shaltiel et al., 2004). MIP-synthase expression in brain is confined to the vasculature (Wong et al., 1987), so it is unclear whether this enzyme contributes to the inositol-reversible effects of the mood stabilizers on neurons (Williams et al., 2002).

Another proposed target for the effects of mood stabilizers on inositol levels in the brain is the sodium-dependent *myo*-inositol transporter (SMIT) (van Calker and Belmaker, 2000). All three

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mood stabilizers affect high-affinity inositol transport in astrocytes by decreasing levels of mRNA encoding SMIT1 (Lubrich and van Calker, 1999). In the mature brain, the highest level of expression of SMIT1 is in the choroid plexus (Guo et al., 1997), although SMIT1 mRNA expression is increased in neural tissue following brain injury (Guo et al., 1997). There is a second sodium-dependent *myo*-inositol transporter, SMIT2, that is widely distributed in several tissues including the brain (Coady et al.. 2002). Uldry et al. (2001) recently described an H^+-dependent *myo*-inositol transporter, HMIT, with a more restricted distribution that is expressed in several brain regions including neurons of the frontal cortex. Interestingly, HMIT is inserted into the neuronal plasma membrane from a vesicular compartment in an activity-dependent manner, suggesting that HMIT may be involved in regulating neuronal PIns synthesis (Uldry et al., 2004).

Lithium also inhibits GSK3 directly leading Klein and colleagues to suggest that GSK3 may instead be the therapeutically relevant target for lithium in mood stabilization rather than PIns signaling (Klein and Melton, 1996; O'Brien et al., 2004; Phiel and Klein, 2001). There are several reports that VPA also inhibits GSK3 either directly or indirectly (Chen et al., 1999; Hall et al., 2002; Kim et al., 2005; Werstuck et al., 2004, De Sarno et al., 2002), but there are other reports that VPA does *not* inhibit GSK3 either directly or indirectly (Jin et al., 2005; Phiel et al., 2001; Ryves et al., 2005; Williams et al., 2002). Another direct target for VPA is histone deacetylase (HDAC), whose inhibition results in changes in neuronal structure due to an increase in β-catenin mRNA and protein levels (Phiel et al., 2001).

Following our report on the inositol-reversible effects of mood stabilizers on rat sensory neuron growth cones, others have suggested that the effects of lithium and VPA on growth cones are instead mediated by inhibition of GSK3. For example, Owen and Gordon-Weeks (2003) reported that the GSK3 inhibitor SB-216763 and 10 mM lithium increased the growth cone spread area of chick sensory neurons. While studying the effects of Wnt signaling on neural development, Salinas and colleagues found that both lithium and VPA increased the spread area of growth cones of cerebellar mossy and granule neurons (Hall et al., 2000, 2002; Lucas and Salinas, 1997), but these drug effects were not reversed by inositol. Guidance cues such as semaphorins, which are involved in directing axons to or away from particular targets, can induce a dramatic 'collapse' of growth cones; the axons retract and do not elongate while semaphorin is present. Semaphorin 3A-induced 'collapse and retraction' behavior is dependent on activation of a pool of inactive GSK3 at the leading edge of the sensory neuron growth cones, which was elegantly demonstrated by Eickholt et al. (2002) using the GSK3 inhibitors SB-216763 and SB-415286 as well as 20 mM lithium. In discussing the evidence that the relevant therapeutic target of lithium in the treatment of bipolar disorder is either inhibition of GSK3 or inhibition of PIns signaling, O'Brian et al. (2004) make no distinction between semaphorin-induced 'collapse and retraction' and the cycles of dynamic 'collapse and spread' we described (Williams et al., 2002). In addition, O'Brien et al. (2004) comment that there may be additional functions of inositol other than the known effects on PIns recycling because addition of *myo*-inositol can reverse the developmental defects induced by dominant-negative GSK3β in *Xenopus* (Hedgepeth et al., 1997).

Our study on growth cones (Williams et al., 2002) is the only example of effects of all three mood stabilizers—lithium, VPA, and CBZ on a common intracellular signaling pathway in neurons. Given the importance of determining the therapeutically relevant targets of these drugs, we sought to clarify the above points and to further characterize this assay.

Results

In further experiments to test whether the growth cone assay is a useful model for mood stabilizing drug action, we labeled growth cones and scored them as collapsed or spread as illustrated in Fig. 1a. Using this assay, we confirmed our previous findings that lithium inhibits the frequency of collapse, and that this effect is reversed by the addition of 1 mM inositol to the tissue culture medium (Fig. 1b). To determine the specificity of this morphological assay, we then tested gabapentin (GPT) (50 μM) and phenytoin (PTN) (50 μM). There was no effect of either drug on collapse (Fig. 1c). These results show that two anti-convulsants that are not effective as anti-manic drugs (Yatham et al., 2002) do not induce inositol-reversible effects on sensory neuron growth

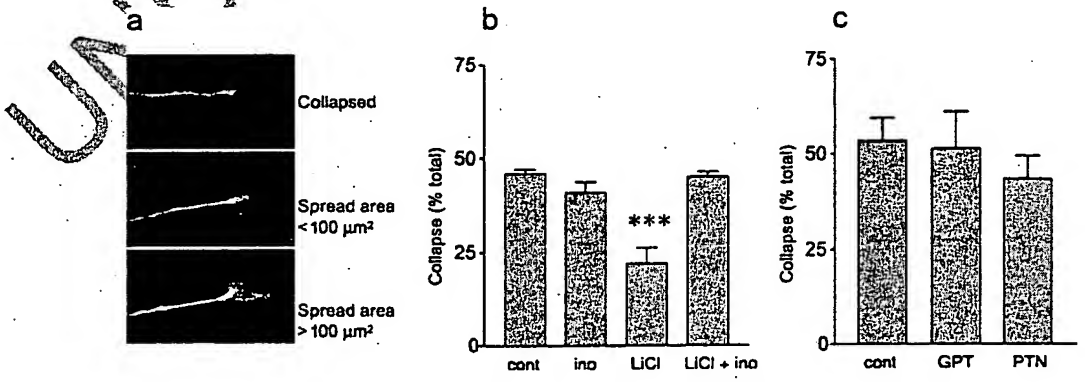


Fig. 1. (a) Micrographs showing a collapsed growth cone (top) and spread (middle and low panels). Sensory explants were labeled with calcein and fluorescent images acquired with a ×40 objective using an Olympus BX51 microscope. (b) Lithium and inositol effects on growth cone collapse in sensory neurons. Histogram shows the effect of LiCl (3 mM) with/without inositol (1 mM) on the number of growth cones collapsed expressed as a percentage of total. In this and the following figures, data are presented as mean ± SEM. Significant change from control is indicated (one-way ANOVA and Fisher's LSD test, $^{***}P < 0.001$ in LiCl-treated explants). Results shown are from two independent experiments each in duplicate ($n = 4$). (c) Gabapentin (GPT) and phenytoin (PTN) do not mimic a mood stabilizer in sensory neuron growth cones. Histogram shows the percentage of growth cones collapsed in cultures treated with GPT 50 μM or PTN 50 μM. Results are from one representative experiment ($n = 3$ coverslips). This experiment was repeated three times with similar results.

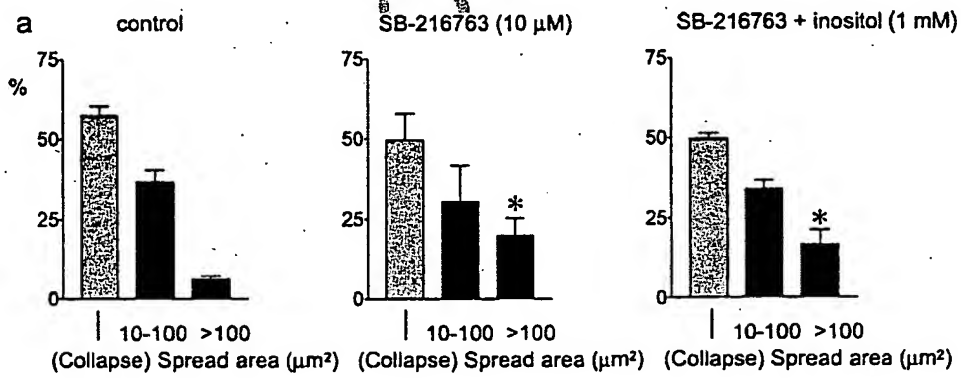
cones and therefore do not mimic the mood stabilizers lithium, VPA, or CBZ.

To determine whether GSK3-mediated effects on growth cones are sensitive to inositol levels as suggested by Klein and colleagues from studies in *Xenopus* (Hedgepeth et al., 1997; O'Brien et al., 2004), we treated sensory neuron explants with the potent GSK3 inhibitor SB-216763 (10 μM), with or without addition of extracellular inositol (1 mM). SB-216763 treatment did not change the percentage of collapsed growth cones (Fig. 2a), but it did increase the percentage of large growth cones with area >100 μm^2 (control: 6 ± 3%, SB-216763-treated: 20 ± 5%; mean ± SEM). There was no difference when inositol was added with the inhibitor (SB-216763 plus inositol-treated: 17 ± 5%) (Fig. 2a). There was a significant increase in the mean spread area of growth cones; for those with areas <100 μm^2 or >100 μm^2, the increases in drug-treated explants were ~40% and ~75%, respectively (Fig. 2b). The mean spread area of all growth cones in control was 53 ± 4 μm^2, and this area doubled in the presence of SB-216763. Importantly, the addition of 1 mM inositol together with the GSK3 inhibitor did not change significantly the mean growth cone area (96 ± 18 vs. 89 ± 17 μm^2) as shown in Fig. 2b. Other GSK3 inhibitors were also tested: SB-415286, GW784752× (Boucheron et al., 2004) and GSK3 inhibitor VIII (Bhat et al., 2003). Qualitative analysis showed that each of these inhibitors induced an increase in the number of large growth cones, but again, there was no difference when inositol was added with the inhibitor (data not shown). Our results show that the effects of mood stabilizers reported in Williams et al. (2002) and in this present study are not mimicked by GSK3 inhibitors, thus ruling out GSK3 as the common target for the mechanism of action of these drugs on sensory neuron growth cones.

We also analyzed the effect of mood stabilizers on rat primary neurons derived from the cerebral cortex, a brain region that has been implicated in the pathophysiology of mood disorders. Lithium (2 mM), VPA (1 mM), and CBZ (25 μM) each increased the percentage of growth cones with area >140 μm^2 (control: 4%, lithium: 43%, VPA: 26%, and CBZ: 67%), with a concomitant reduction in the percentage of growth cones with area comprised between 20 and 140 μm^2 (control: 90%, lithium: 56%, VPA: 71%, CBZ: 41%) (Fig. 3). Addition of inositol (1 mM) completely or partially reversed this increase in growth cone area induced by the drugs in a similar way to that observed in sensory neurons (Fig. 3). These results show that the common inositol-reversible effects of mood stabilizers on growth cone area that we observed in sensory neurons can be replicated in neurons that are more relevant for mood disorders.

We then analyzed the effects of SB-216763 on cortical neurons. This GSK3 inhibitor induced the formation of large growth cones with area >140 μm^2 (control: 3%, SB-216763-treated neurons: 19%), and the addition of inositol did not reverse this effect (SB-216763 plus inositol-treated cultures: 21%) (Fig. 4). These results confirm our findings with sensory neurons showing that the increase in growth cone area induced by the GSK inhibitor SB-216763 is not reversed by inositol.

To further rule out the involvement of GSK3 in the common mechanism of action of mood stabilizers, we analyzed the effects



b

	Collapse (% total)	Mean area (μm² ± sem) <100	>100	all
control	57 ± 3	41 ± 2	131 ± 8	53 ± 4
SB-216763	50 ± 11	58 ± 6	229 ± 41	96 ± 18
SB-216763 + inositol	50 ± 2	52 ± 3	182 ± 17	89 ± 17

Fig. 2. (a) SB-216763 (10 μM) increases sensory neuron growth cone area and inositol does not reverse the effect. Histograms show the percentage of sensory neuron growth cones that were collapsed or had spread areas between 10–100 μm^2 and >100 μm^2 in control or SB-216763-treated cultures with/without inositol (1 mM), as indicated. *$P < 0.05$ in SB-216763-treated cultures in the population of growth cones with area >100 μm^2 when compared with control (ANOVA and Fisher's LSD test). Results were pooled from three independent experiments ($n = 3$ coverslips each). The total number of growth cones scored in each bar was ~300. (b) SB-216763 increases the growth cone spread area values when compared with control. The table shows the percentage of collapse and growth cone area values for control or SB-216763-treated cultures with/without inositol (mean ± SEM). Data are from the same experiment shown in panel a.

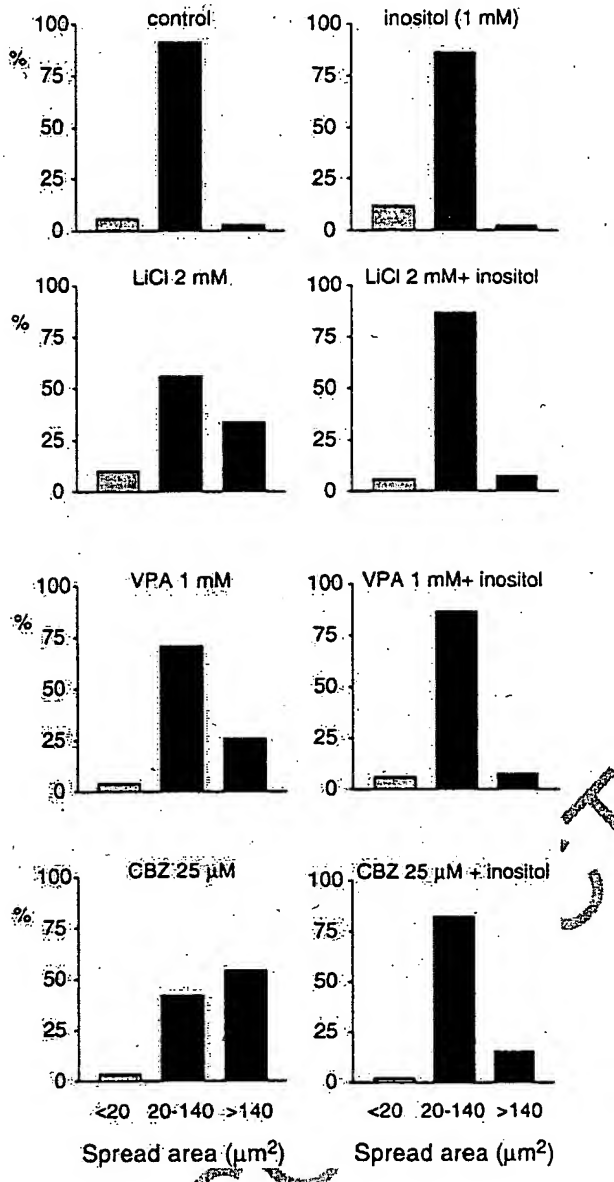


Fig. 3. Effect of mood stabilizers on growth cone area in cortical neurons. Histograms show the percentage of spread growth cones after treatment with drugs with/without the addition of inositol, as indicated. LiCl, VPA, and CBZ each induced a shift to larger areas and addition of inositol completely or partially (CBZ) reversed the drug effects. Data are from one experiment ($n = 3$ coverslips, pooled data), and the total number of growth cones scored in each bar was ~100–150. The experiment was repeated with similar results. Note that the cortical neurons do not have a cycle of complete collapse and so the first bin shows small growth cones with area <20 µm².

of the drugs on intracellular proteins activated/inhibited by GSK3-dependent phosphorylation. Cortical neurons were treated after 5 days in culture with two different concentrations of mood stabilizers for 6 h (1 and 10 mM lithium chloride, 1 and 2 mM VPA, 50 and 100 µM CBZ; in each case, the lower dose is close to the therapeutic range for blood levels of the drugs). We used Western blotting to study glycogen synthase (GS), tau, and β-catenin expression. GS is a well-characterized GSK3 substrate, and it is inactivated by GSK3-mediated phosphorylation. Phospho-GS protein levels were reduced to nearly undetectable levels after treatment with high concentrations of lithium chloride (10 mM) or SB-216763 (10 µM) (Figs. 5a, b). In contrast, lithium chloride at the therapeutic concentration of 1 mM, as well as VPA (1, 2 mM), and CBZ (50, 100 µM) did not have significant effects on phospho-GS levels. Likewise, only 10 mM lithium and SB-216763 decreased phosphorylation levels of the neuronal-specific microtubule-associated protein tau (assessed using the monoclonal antibody (mcAb) AT8 that recognizes the tau/phosphoserine$_{202}$ epitope), while both lithium and SB-216763 increased dephosphorylated tau levels (assessed using mcAb Tau-1). These results are consistent with lithium (but not VPA or CBZ) inhibiting endogenous GSK3 activity; moreover, the effect of lithium chloride was only seen with the high concentration of 10 mM and not with the therapeutically relevant 1 mM. When we analyzed β-catenin levels, we found that lithium chloride (10 mM), SB-216763 (10 µM), and VPA (2 mM) each increased levels of cytoplasmic β-catenin. The HDAC inhibitor trichostatin A (TSA) also induced a 3-fold increase in β-catenin levels (data not shown), suggesting that the effects of VPA on β-catenin levels are likely due to HDAC inhibition. Our results strongly suggest that only lithium (and not VPA or CBZ) inhibits GSK3 either directly or indirectly. Similar conclusions were drawn in a recent study (Ryves et al., 2005) where only lithium decreased tau phosphorylation (mcAb AT270 was also used). In that study, however, neither lithium nor VPA changed β-catenin levels, whereas we saw an increase in β-catenin levels in response to lithium, VPA and trichostatin A, as predicted from previous work (Phiel et al., 2001). The lack of effect of VPA on β-catenin in Ryves et al. (2005) maybe due to the different extraction procedure. Our data, together with the growth cone results presented above, provide strong support to the idea that the common effects of the mood stabilizers on both sensory and cortical neuron growth cones are mediated by inhibition of PIns signaling and not by inhibition of GSK3 signaling.

We then investigated several genes encoding proteins involved in regulating inositol availability in the central nervous system, namely MIP-synthase and the known *myo*-inositol transporters. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis was performed on mRNA isolated from lysates of cortical neurons cultured for either 5, 7, or 9 days (dic). Rat (postnatal day 21) kidney and brain mRNA were used as positive controls for SMIT and MIP-synthase respectively. We did not detect MIP-synthase, SMIT1 and SMIT2 mRNA expression in cortical neurons (data not shown). In contrast, neurons expressed HMIT mRNA at all days in culture analyzed (Fig. 6a). We also confirmed the presence of HMIT protein in cultured cortical neurons by immunocytochemistry (Fig. 6b). HMIT was expressed in all neurons, both in cell bodies and in neuronal processes, as shown by co-localization with the neuronal marker β-III-tubulin.

Discussion

None of the drugs currently used to treat bipolar disorder were designed or developed with this use in mind. Other than lithium, all of the mood stabilizers were first used as anticonvulsant drugs for the treatment of epilepsy. Lithium, VPA, and CBZ are each effective in the control of mania, and both lithium and VPA are also effective at limiting the frequency of mood swings to depression. Although the mood stabilizers are effective drugs for treating bipolar disorder, their various side effects limit their acceptability as maintenance treatment, and there is a clear and

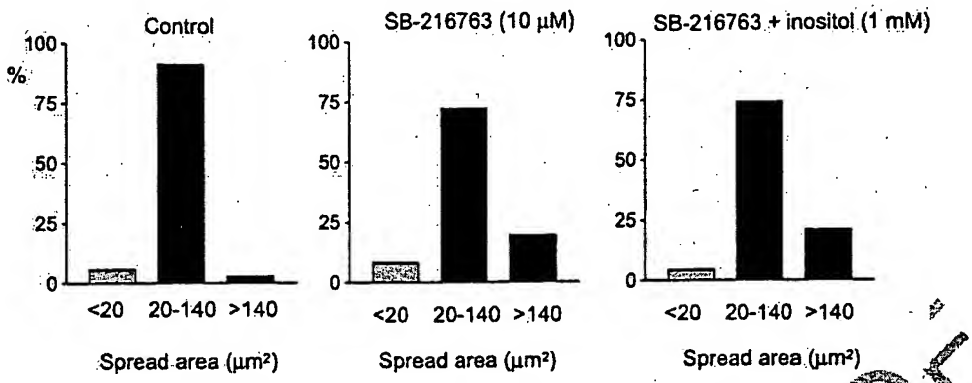


Fig. 4. SB-216763 (10 μM) increases cortical neuron growth cone spread area and inositol does not reverse the effect. Histograms show the percentage of different size cortical neurons growth cones treated with SB-216763 with/without inositol. Data are from one experiment (n = 3 coverslips pooled data). The total number of growth cones scored in each bar was ~100–150. The experiment was repeated with similar results.

urgent need to develop more efficacious and better tolerated drugs. This aim is greatly hampered by the lack of useful animal models for bipolar disorder and no useful drug screens for testing potential candidate drugs. The growth cone assay we describe in this paper and previously (Williams et al., 2002) is the first to show a common effect on neurons of the three most widely prescribed drugs for maintenance treatment, and therefore, it is potentially useful for assessing new candidate drugs. In this study, we addressed some controversial issues about the assay and further investigated the role of the PIns signaling pathway in the common action of lithium, VPA, and CBZ on neurons.

We find that gabapentin and phenytoin, anticonvulsant drugs that are not commonly used to treat bipolar disorder (Yatham et al., 2002), do not mimic the effects of the mood stabilizers on sensory neuron growth cones. Thus, the action of the anti-convulsants on growth cones parallels their clinical efficacy in the treatment of bipolar disorder rather than their efficacy in the treatment of epilepsy. We also show that lithium, VPA, and CBZ each increase the spread area of growth cones of neurons derived from the rat cerebral cortex, and these effects are reversed by inositol. While it is important that the drugs work in a similar fashion on neurons from a brain region known to be involved in mood control, sensory neurons offer several advantages over cortical neurons for assay, e.g., directionality of axon growth and a cycle of complete 'collapse' as they continue to grow, which can be easily scored as a percentage of total growth cones.

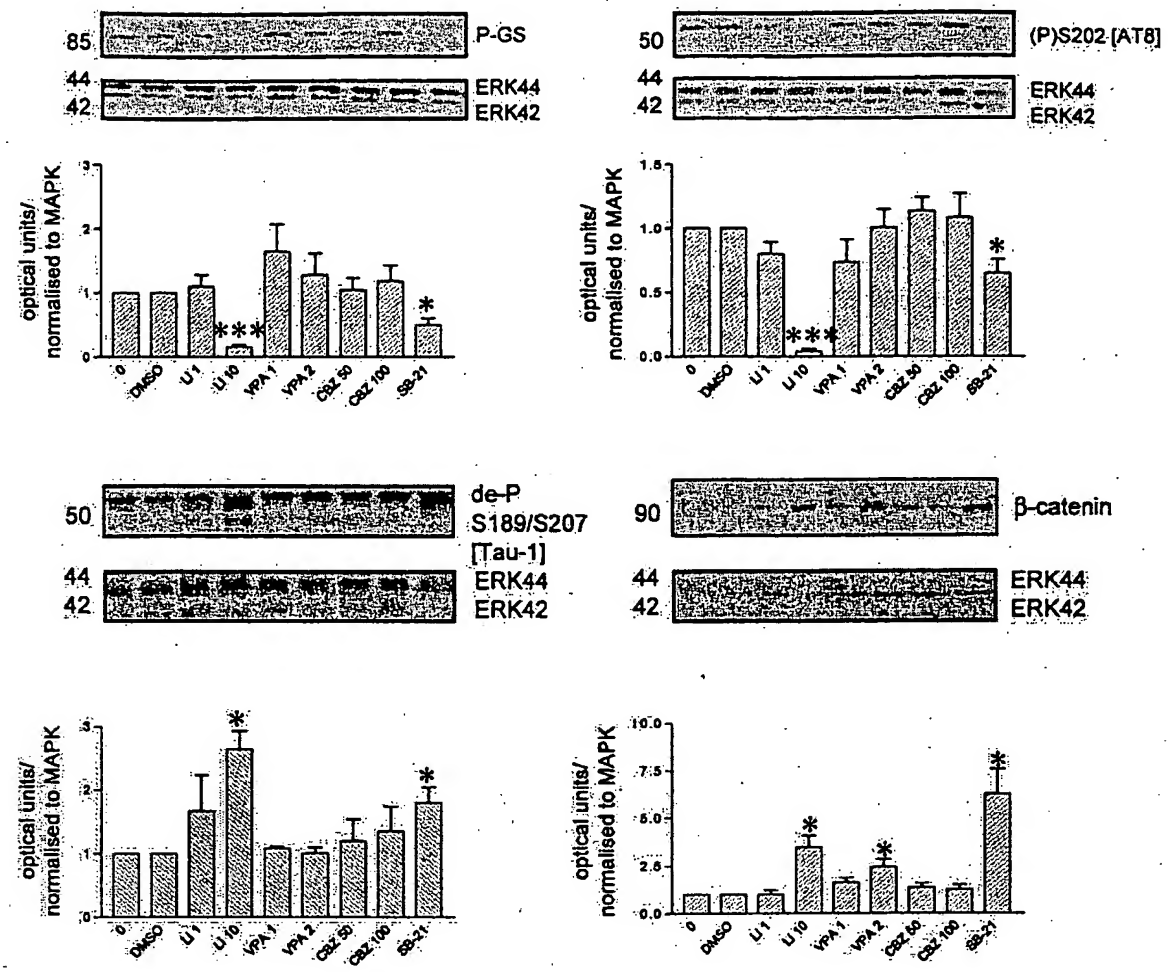
In both sensory and cortical neurons, the GSK3 inhibitor SB-216763 induced a population of very large growth cones (>100 μm^2), some of which had microtubules abnormally penetrating into the growth cone (data not shown). Addition of inositol did not change the percentage of such large growth cones, nor did inositol reverse the increase in mean spread area induced by the inhibitor in the growth cones in the size range of 10–100 μm^2. (Note that the latter population generally did not contain microtubules abnormally penetrating into the growth cones). These results are in contrast to the report by Hedgepeth et al. (1997) that inositol reversed the effect of GSK3 ablation in developing *Xenopus*. Since we used inositol reversibility to argue that the common effect of the mood stabilizers on growth cones was mediated by inhibition of PIns signaling (Williams et al., 2002), our results showing that inositol does not reverse the effects of GSK3 inhibition on growth cones are important and add further support to our previous conclusion.

In our previous study in sensory neurons (Williams et al., 2002), we commented that there was a small population (<2%) of very large growth cones with penetrating microtubules in the presence of 10 mM (but not 3 mM) lithium, but these did not contribute significantly to the growth cone spread area data. In contrast, Owen and Gordon-Weeks (2003) describe many more abnormal growth cones in 10 mM lithium-treated embryonic (E8) chick neurons. The E7–E8 chick neurons would still be at the stage where axons are using guidance cues such as semaphorin 3A to navigate to their targets, whereas the postnatal rat neurons used in both our studies would have reached their peripheral targets prior to explanting. Therefore, axon rapid growth in the postnatal neurons would be of a regenerative nature where axons use laminin in Schwann cell basement membranes to navigate. Interestingly, GSK3 expression is downregulated at the end of axonogenesis (Leroy and Brion, 1999). Because semaphorin 3A signaling involves activation of an inactive pool of GSK3 in the growth cone, it may be that there is a difference in the levels of GSK3 in the embryonic vs. postnatal neurons that could explain whether lithium produces more or less GSK3-dependent effects on the growth cone spread area.

It is also notable that chick sensory neurons have larger growth cone areas in the control than do the postnatal rat neurons (~150 vs. 50 μm^2). Likewise control cerebellar neurons (both mossy and granule) used by Salinas and colleagues to study Wnt signaling (Hall et al., 2000, 2002; Lucas and Salinas, 1997) were also large (~200 μm^2) compared with both the rat sensory and cortical neurons we studied. Our interest is in determining the mechanism of action of the drugs, and we use the sensory neurons as a morphological read-out of intracellular signaling events. In contrast, those interested in growth function likely choose to study neurons with large growth cones that do not collapse spontaneously and where the effects of GSK3 inhibition may be greater. (Time lapse movies of growth cones treated with mood stabilizers can be viewed on http://www.ucl.ac.uk/lmcb//anne/Mudge/MechanismOfMoodStabilizers.html).

There is much controversy regarding the question of whether VPA inhibits GSK3 either directly or indirectly. We show in our study that only lithium, and not VPA, affects phosphorylation of known GSK3 targets in cortical neurons in agreement with Jin et al. (2005), Phiel et al. (2001), Ryves et al. (2005), Williams et al. (2002). In contrast to the reports by Williams et al. (2002) and Ryves et al. (2005), however, we found that both VPA and lithium increased the levels of β-catenin; the VPA effect was mimicked by trichostatin A suggesting that VPA increases β-catenin levels by inhibiting HDAC in cortical neurons, as predicted by previous

a



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	P-GS	AT8	TAU-1	β-catenin
LiCl (10 mM)	↓ 0.15 ± 0.04 (***p<0.001)	↓ 0.04 ± 0.02 (***p<0.001)	↑ 2.64 ± 0.29 (**p=0.01)	↑ 3.47 ± 0.62 (*p<0.05)
VPA (2 mM)	No change	No change	No change	↑ 2.44 ± 0.41 (*p<0.05)
CBZ	No change	No change	No change	No change
SB-216763	↓ 0.50 ± 0.11 (*p<0.05)	↓ 0.65 ± 0.11 (*p<0.05)	↑ 1.80 ± 0.25 (*p<0.05)	↑ 6.25 ± 1.34 (*p<0.05)

Fig. 5. (a) Lithium inhibits GSK3 activity in cortical neurons. Western blotting analysis of cortical neurons treated with drugs for 6 h. (a) A representative blot is shown, and the asterisk indicates a significant difference compared with control. (b) Quantitative changes of GSK3 downstream targets expression after treatment with mood stabilizers or with the GSK3 inhibitor SB-216763. Data were quantified from at least three independent experiments and normalized to ERK/MAPK for sample loading. Increase in protein expression when compared with control is indicated with ↑ and decrease with ↓. Mean ± SEM is shown. Single sample *t* test was used in the analysis, and the *P* value is given when the effect was statistically significant.

work (Phiel et al., 2001). It is likely that VPA inhibition of HDAC is responsible for the teratogenic effects of VPA (Gurvich et al., 2004).

Both MIP-synthase and the inositol transporters SMIT1 and SMIT2 have been suggested as therapeutically relevant targets for mood stabilizers, but our finding that cortical neurons do not express the genes encoding these proteins makes it clear that they play no part in the inositol-reversible effects of the drugs in cortical neurons. The location of MIP-synthase in brain endothelial cells and the high concentration of SMIT1 in the choroid plexus make it likely, however, that they are both involved in regulating the level of inositol in the brain, which is ~10-fold

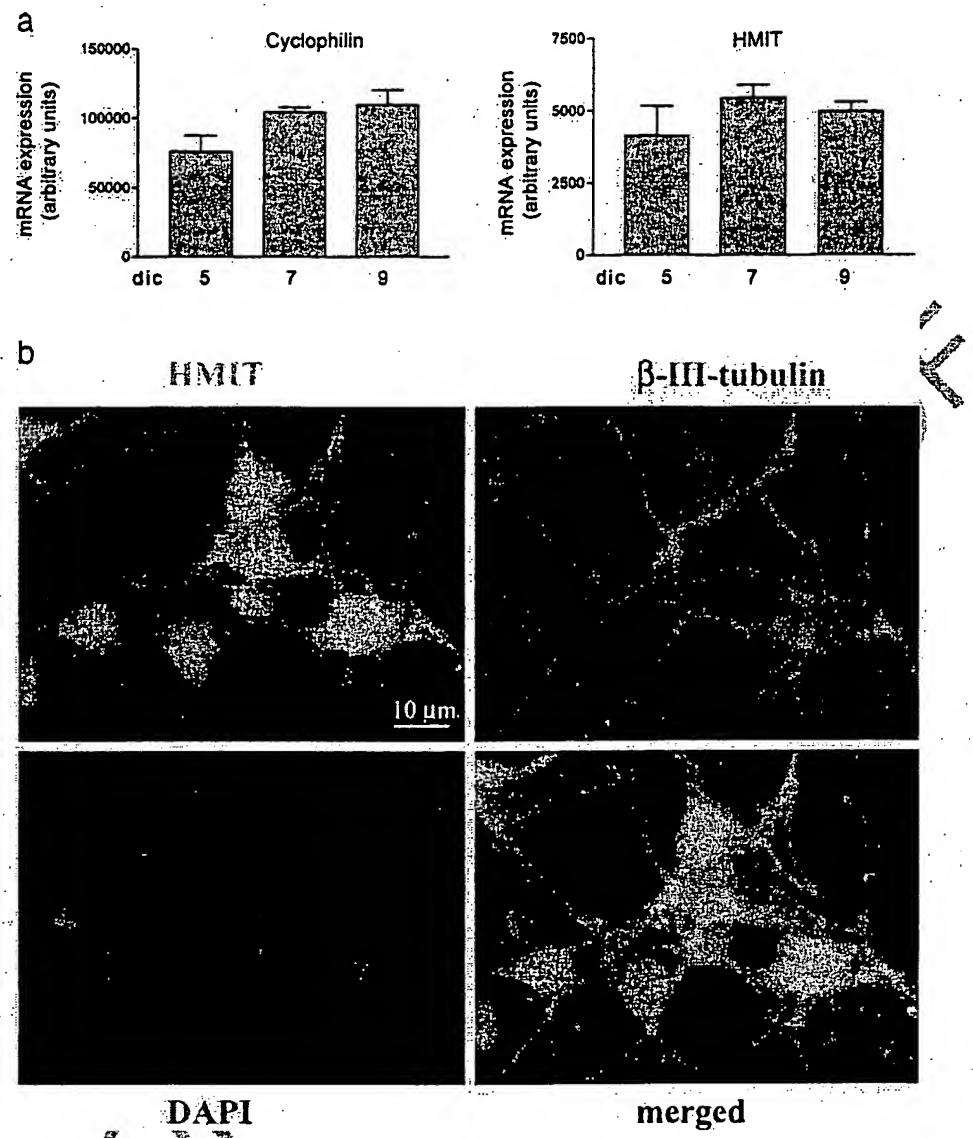

Fig. 6. (a) Cortical neuron mRNA analysis. TaqMan RT-PCR analysis of primary cortical neurons at 5, 7, and 9 days in culture shows expression of HMIT mRNA at each time in culture. Cyclophilin was used as housekeeper control gene. (b) Cortical neurons express HMIT protein. Fluorescent micrographs show HMIT immunolabeling in cell soma and neurites (green). HMIT colocalizes with the neuronal marker β-III-tubulin (red). Cell nuclei are labeled with DAPI. Images were acquired with an epifluorescence microscope using a ×100 oil lens.

higher than in blood (Eisher et al., 2002). Interestingly, both VPA and lithium increase the expression of the MIP-synthase gene homologue *ino-1* in yeast (Ju et al., 2004; Vaden et al., 2001) and *Dictyostelium* (Williams et al., 2002), but it seems that neurons have evolved different mechanisms for regulating the availability of this important precursor for membrane PIns synthesis. Berry and colleagues reported that the fetal brains of mice with a SMIT1 gene deletion had normal levels of inositol phospholipids (Berry et al., 2003, 2004), and they raised the question of how neurons obtain their inositol for PIns synthesis. Our finding that the cortical neurons express HMIT, but not SMIT1 or 2, starts to address this question. HMIT recently described by Uldry et al. (2001) is expressed predominantly in the brain, suggesting a unique role in regulating brain inositol metabolism. The cellular localization of HMIT is regulated by neuronal activity, suggesting it may link the supply of inositol to activity of the PIns cycle (Uldry et al., 2004). HMIT is present in subsets of neurons in the cerebral cortex, an area implicated in mood control (Uldry et al., 2001, 2004). Our results showing that HMIT is expressed in cultured cortical neurons, whereas SMITs and MIP-synthase are not, suggest that HMIT is likely to play a crucial role in regulating both the supply of inositol for PIns synthesis and the action of mood stabilizers.

Taken together, our present results confirm and extend our previous conclusion that the common effects of the three mood stabilizers on neurons are mediated by inhibition of PIns signaling, and that these inositol-reversible effects do not involve GSK3 inhibition. Moreover, the growth cone assay should be useful for testing potential drugs for their ability to mimic lithium, VPA, and CBZ on inositol-reversible signaling in neurons, independently of GSK3, SMIT, or MIP-synthase. Our results also raise interesting questions about how cortical neurons regulate levels of intracellular inositol, which is crucial for PIns synthesis and thus the function of the PIns signaling pathway.

Experimental procedure

Drugs

Drugs were obtained from the following sources: lithium chloride, VPA, CBZ, *myo*-inositol (referred to in the text as inositol), trichostatin A, gabapentin were from Sigma; SB-216763, SB-415286, GW784752X were synthesized and supplied by GlaxoSmithKline. Phenytoin and the GSK3 inhibitor VIII were from Calbiochem.

Phospho-glycogen synthase antibody preparation

This antibody was generated and validated at GlaxoSmithKline by Western blotting and ELISA and shown to recognize the phosphorylated form only. The GS peptide sequence used to generate antibodies was QGYR-YPRPAS(PO3)VPS(PO3)PSLSRHSSPHQSEDEEDPR corresponding to site 3a and 3b of GS (Tony Wang, personal communications).

Dorsal root ganglia explants

Dorsal root ganglia (DRG) from P0–P2 rats (Charles River, UK) were dissected and cultured as explants on coverslips coated with poly-D-lysine (M.W. >300,000, Sigma) and laminin (used at 50 μg/ml) (Sigma) in serum-free media with nerve growth factor at 50 μg/ml (Invitrogen) as previously described (Cheng and Mudge, 1996; Williams et al., 2002). Drugs were added after 20 h in culture, and explants were cultured for a further 16–24 h before loading the neurons with calcein dye (Molecular Probes). Note that the method followed is a slight modification from Williams et al. (2002), in that the timings are different.

Primary cortical neurons

Primary cortical neurons were dissociated from embryonic day 18 Sprague–Dawley rat (Charles River, UK) cerebral cortices as previously described (Di Daniel et al., 2005) in accordance with the 1986 UK Animals Act (Scientific Procedures). Neurons were seeded at 500,000 cells/well into 6-well plates coated with poly-D-lysine (M.W. >300,000, Sigma) and then treated after 5 days in culture (5 dic) with the drugs for 6 h. For growth cone morphological studies, neurons were plated at 12,500 cells/well on poly-D-lysine coated coverslips in 24-well plates and treated with drugs 20 h after plating for 4 days. For mRNA analysis, neurons were lysed at 5, 7, or 9 dic.

Immunocytochemistry

Sensory neurons

Explants were loaded with the fluorescent dye calcein for 20 min at 37°C and then fixed with 4% (v/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde for 30 min. Coverslips were mounted with Citifluor (Citifluor). *Cortical neurons*: Neurons were fixed with 4% (v/v) paraformaldehyde for 15 min and permeabilized with 0.3% Triton X-100 for 5 min. Cells were then blocked with 5% (v/v) normal goat serum (Chemicon) in phosphate-buffered saline (PBS) (Invitrogen) for 1 h and then incubated with the polyclonal anti-GAP-43 antibody (kindly provided by G. Wilkinson, Imperial College London, used at 1:1000) overnight at 4°C in PBS with 5% normal goat serum or with the polyclonal anti-HMIT antibody (Alpha Diagnostic International, used 1:100) overnight at 4°C or with the monoclonal β-III-tubulin (Sigma, used 1:3000). A secondary Alexa-conjugated anti-rabbit or anti-mouse IgG antibody (Molecular Probes) was added after 3 washes with PBS for 1 h. Coverslips were mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes).

Analysis of sensory and cortical neuron growth cone areas

Random fields at the perimeter of the axonal halo that grew out from the explant cell bodies were scored using a X40 objective and an Olympus BX51 microscope. The perimeter of calcein-labeled growth cones was traced with a light pen, and the area was calculated using the Image ProPlus software. Experiments were performed in triplicate for each treatment, and measurements were pooled from up to four independent preparations to generate the data shown. Images of cortical neurons were acquired from 10 random fields with a ×20 objective. For each drug treatment, ~100–300 growth cones were measured in each experiment. The experiments were repeated as described in the legends.

Western blotting

Neurons were washed with cold PBS and lysed in sample buffer (2% (w/v) SDS, 10% (v/v) glycerol, 25 mM TRIS pH 6.8, bromophenol blue, 1% (v/v) β-mercaptoethanol). For β-catenin extraction, a detergent-free, hypotonic buffer was used (Cross et al., 2001). Equal amounts of protein lysates were resolved by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% gels (Novex, Frankfurt, Germany) and immunoblotted to nitrocellulose membranes (Amersham, Buckinghamshire, UK) as previously described (Di Daniel et al., 2005). The following primary antibodies were used: mcAb anti-tau dephosphorylated on Ser189/207 (clone Tau-1, Chemicon, used at 1:1000), mcAb anti-tau phosphorylated on Ser202 (clone AT8, Autogen Bioclear, used at 1:1000), mcAb anti-β-catenin (Chemicon, used at 1:1000), mcAb anti-phospho-glycogen synthase (used at 1:500), rabbit polyclonal Ab anti-mitogen-activated protein kinase (ERK1/2) (Upstate, used at 1:10,000). Protein levels were normalized to ERK1/2, and quantification was performed using "GeneTools" software from Syngene. Experiments were repeated at least 3 times. Single sample *t* test was used in the statistical analysis.

TaqMan RT-PCR

Neurons were washed with PBS and lysed with 500 μl of RLT buffer (Qiagen) containing 1% (v/v) β-mercaptoethanol. Samples were homogenized using the MixerMill MM 300 (Qiagen) 2 × 2 min at 30 Hz. RNA extraction was performed using Qiagen RNeasy Mini Kit following manufacturer's instructions including the DNase digestion step (RNase-DNase kit, Qiagen). The same procedure was used to extract RNA from rat cortex and kidney (both from postnatal day 21). RNA quality was determined by measuring the absorbance at 260 and 280 nm. All samples showed 260/280 ratio >1.8. Reverse transcription reactions to generate cDNA from RNA were performed in triplicate including a negative control (minus enzyme) following manufacturer's instructions (Qiagen Omniscript Reverse Transcriptase Kit). Oligo (dT)12–18 and RNase out ribonuclease inhibitor were from Invitrogen. TaqMan analysis was performed using the Applied Biosystems ABI 7700 PE machine (Foster City, CA), as previously described by Medhurst et al. (2000). Briefly, each reaction contained 1

Table 1
Taqman primer and probe sequences

Gene name/accession no.	Fw primer (5–3)	Rev primer (5–3)	Probe (5–FAM, 3-TAMRA) (5–3)
SMIT1 NM_053715	ATGAAGACGTCCCATGGCCT	CCCTCTGCACGATGACTTGG	ATTCATTCTTGGGCAGACCCCAGCCTC
SMIT2 Prediction XM_574554.1	TCCTGTGGCTCTGTGGGATG	TGCACACAATGCAGTTGACG	CCCCAAGCAAAGTGGAGCCTGTCATAG
MIP-synthase NM_001013880	AAGATGGAGCGCCCTTTCC	AGCTCTGGTGTTGGTGCCTG	CCACTGCCTTGCAAGAAAGAGTCCACA
HMIT NM_133611	TCGAATCGCTCTTCGACCAC	CCTTCACGCGGATGTACTCG	CGGACTCGGACGAGGGCAGGTACAT
Cyclophilin M19533	TGTGCCAGGGTGGTGACTT	TCAAATTTCTCTCCGTAGATGGACTT	CCACCAGTGCCATTATGGCGTGT

µl cDNA, 11.25 µl mastermix-Excite 2× probe mastermix (GeneSys Ltd., Camberley, Surrey), 11.25 µl of water, 0.5 µl of forward primer (synthesized by Invitrogen), 0.5 µl of reverse primer (synthesized by Invitrogen) and 0.5 µl of TaqMan FAM-TAMRA labeled probe (synthesized by Operon Biotechnology GMBH). The PCR amplification was performed for 40 cycles, consisting of 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The Ct values were converted to relative copy number using the formula described in Harrison and Bond (2005). Table 1 shows the primer/probe sequences used.

Statistical analysis

Statistical analysis was performed using Statistica v6. Results were considered significant if $^*P$ values were <0.05. Separate one-way ANOVAs were performed on the percentage data for the collapsed, 10–100 µm^2 and >100 µm^2 growth cones. Comparison of treatment groups was made using Fisher's LSD test.

Acknowledgments

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